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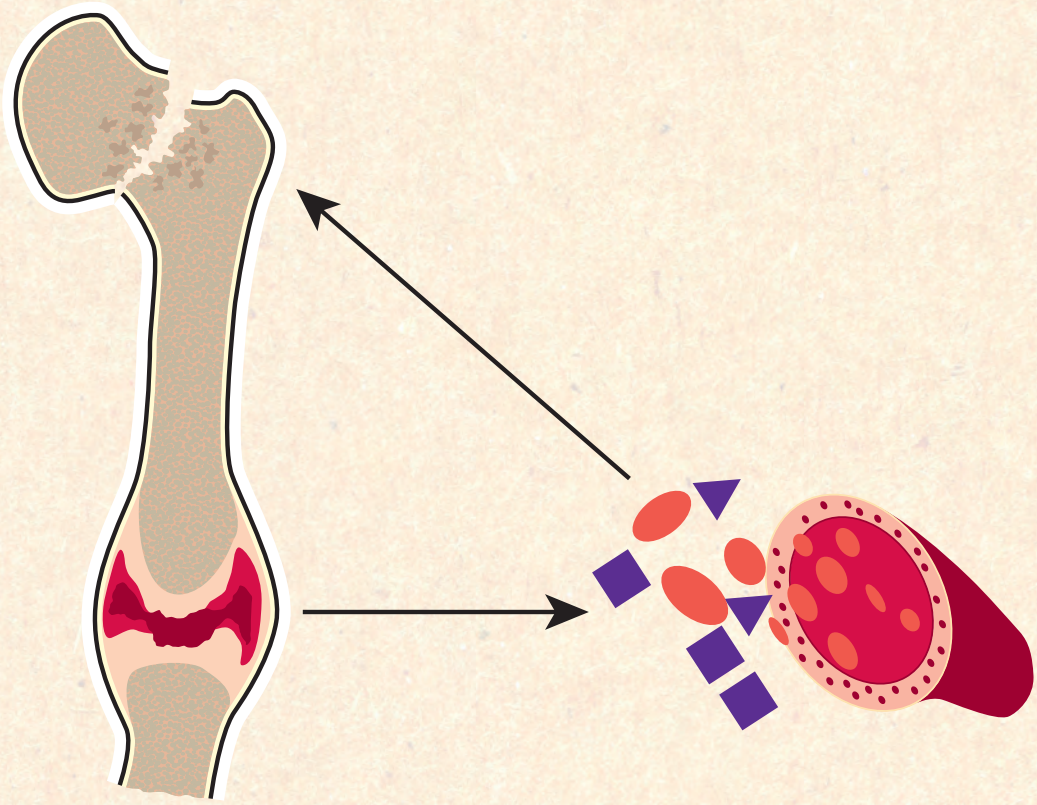

Janak L. Pathak

The Etiology of Generalized Osteoporosis in Rheumatoid Arthritis

Janak Lal Pathak

The studies described in this thesis were carried out at the section Oral Cell Biology of the Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, MOVE Research Institute Amsterdam, The Netherlands, and at the Skeletal Biology and Engineering Research Center, KU Leuven, Leuven, Belgium, with funding by the European Commission through Move-Age, an Erasmus Mundus Joint Doctorate programme (2011-0015).

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CONTENTS

CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown etiology, characterized by painful inflammation of joints, local bone erosion, joint space narrowing, and extra-articular manifestation such as generalized osteoporosis (1, 2). Early stage RA patients show decreased bone mineral density, and a two-fold increase in risk of vertebral and non-vertebral (including hip) fractures compared with healthy controls (3-6). Such fractures are associated with severe morbidity and an increased mortality rate (7). Generalized osteoporosis therefore represents a serious complication of RA. However the main cause of generalized bone loss in RA is still unknown.

Generalized osteoporosis in RA is caused in part by immobility and prolonged high dose corticosteroid therapy (8, 9). It also has been attributed to the effects of chronic systemic inflammation, such as elevated levels of circulating cytokines (10, 11). Tumor necrosis factor α (TNFα) antibodies have been shown to decrease systemic bone loss and increase bone mineral density, indicating that elevated levels of TNFα in RA are associated with osteoporosis and osteopenia (12, 13). Inversely, deletion of the interleukin-1 receptor antagonist leads to the development of arthritis with cartilage destruction and bone destruction (14, 15). Wnts and Wnt antagonists play a crucial role in bone homeostasis via their effect on osteogenesis (16). Activation of canonical Wnt signaling exclusively in osteocytes induces bone anabolism and triggers Notch signaling (17). A crucial role for canonical Wnt signaling has been established, but much remains to be discovered with respect to its fine tuning and crosstalk with other pathways in bone, and the effect of systemic inflammation on such crosstalk (18). The mechanism of the effect of systemic inflammation on generalized bone loss is however still unclear.

Cells in the inflamed synovia in RA produce high amounts of growth factors, in addition to certain cytokines and chemokines such as interleukin-1β (IL-1β), 1IL-6, IL-17, TNFα, CXCL8 (IL-8), CXCL9, CXCL10, and CCL20 (10, 19-22). Elevated levels of these factors are found in synovial fluid and serum from RA patients (10, 19-22). RA-serum not only contains inflammatory cytokines and/or chemokines, but also a number of other factors, such as anti-inflammatory factors, antibodies, auto-antibodies, receptors, decoy receptors, etc.. Since bone is a well-vascularized tissue, a complex mixture of inflammatory cytokines, chemokines, and other signaling factors present in the circulation as a result of systemic inflammation can easily reach the bone cells. The inflammatory cytokines IL-1β, TNFα, and IL-6 are generally known to cause bone loss, but how a complex mixture of inflammatory cytokines, chemokines, and other signaling factors in the circulation affects bone cell function is still a mystery.

Cytokines and chemokines

Cytokines are small (2-20kDa) cell signaling molecules produced by a broad range of cells, including immune cells like macrophages, B lymphocytes, T lymphocytes, and mast cells, as well as endothelial cells, fibroblasts, osteoblasts, osteocytes, and various stromal cells. The inflammatory cytokines IL-1β, IL-6, IL-17 and TNFα are apart from their involvement in inflammation, are also involved in bone remodeling (10, 11, 23-25). Healthy bone requires physiological concentrations of these signaling molecules, while altered signaling molecule levels can cause disturbance of bone homeostasis (11, 26, 27). Chemokines are chemo-attractant cytokines playing an important role in the immunological tolerance and movement of immune cells (28). Although chemokines are well known to be involved in the pathogenesis of RA by recruiting inflammatory immune cells to the synovium (22), their role in bone remodeling is still unclear.

Effect of cytokines on bone cell formation and function

Osteoblasts, osteocytes, bone lining cells, and osteoclasts are the bone cells involved in bone remodeling. Fully differentiated osteoblasts form bone by producing matrix proteins and aiding matrix mineralization (29). The inflammatory cytokines IL-1β, IL-6, and TNFα, and the growth factors transforming growth factor beta (TGFB), Bone morphogenic protein 2 (BMP2), and BMP7 affect osteoblast differentiation and bone formation (11, 26, 27). However, the effect of individual chemokines, and that of the complex combination of inflammatory cytokines, chemokines, and other signaling factors present in the serum of patients with chronic inflammatory diseases on osteoblast differentiation and function is still unknown. Osteoclasts are multinucleated bone resorbing cells of monocyte origin (30). The inflammatory cytokines IL-1β and TNFα directly stimulate osteoclastogenesis and osteoclast activity (10, 11, 27, 31, 32). Inflammatory cytokines affecting osteoblast and osteoclast formation and activity may cause a disturbance of the delicate balance between bone formation and resorption during bone remodeling (10, 11).

Osteocytes sense mechanical stimuli and deliver signals to osteoblasts and osteoclasts (17, 33-36). Osteocytes also release cytokines and signaling molecules that play a key role in bone remodeling (17, 23, 25, 35, 37). They produce pro-osteoclastogenic signals in the absence of mechanical loading, leading to the stimulation of bone resorption (33). In the presence of mechanical stimuli, osteocytes produce factors that inhibit osteoclastogenesis and/or decrease the production of osteoclast-stimulating signals (34). Recombinant IL-1β and TNFα reduce the physiological response of osteocytes to mechanical stimuli, which disturbs the osteocyte-mediated balance between osteoblast and osteoclast activity (23, 24). Although the primary function of osteoblast is to form bone, and the primary function of osteocytes is to maintain the balance between bone

formation and bone resorption by sensing mechanical stimuli, during bone remodeling, osteoblasts and osteocytes also communicate with osteoclasts via the release of cytokines, e.g. receptor activator of nuclear factor-kappa B ligand (RANKL), osteoprotegerin (OPG), IL-1β, and IL-6 (38, 39). Inflammatory cytokines, such as TNFα and IL-1β, affect cytokine production by osteoblasts in an autocrine manner (38, 39). TNFα, RANKL, OPG, IL-1β, and IL-6 are known to play a vital role in osteoclast formation and activity (38-40). Enhanced levels of proinflammatory cytokines such as TNFα, IL-1β, IL-6, and IL-17 in arthritic joints and in the systemic circulation do not only directly disturb the balance between osteoblastic bone formation and osteoclastic bone resorption (5, 20), but they also affect osteocyte and/or osteoblast communication towards osteoclasts resulting in bone loss (11, 23, 24). However, it is still not fully understood how systemic inflammation affects this osteoblast and/or osteocyte-to-osteoclast communication.

Effect of cytokines on bone regeneration

Osteoblasts are derived from mesenchymal stem cells (MSCs) and play a vital role in bone regeneration. MSCs have the potential to differentiate into multiple mesenchymal cell lineages such as osteoblasts, adipocytes, and chondrocytes (41, 42). Bone marrow, adipose tissue, blood, and periosteum represent a major source of MSCs (43-46). MSCs differentiate along the osteo/chondrogenic pathway during bone healing and regeneration (41, 42). Elevated levels of cytokines/chemokines likely inhibit bone formation during systemic inflammation. IL-1β and TNFα inhibit endochondral bone formation in murine postnatal metatarsal bones, suggesting that these cytokines also inhibit bone healing and regeneration (47). Bone healing and regeneration is delayed during systemic inflammation, which causes a decrease in bone mineral density (48-50). However, the exact influence of the complex combination of inflammatory cytokines, chemokines, and other signaling factors on the differentiation of MSCs along the osteo/chondrogenic pathway is still unknown.

Elevated levels of inflammatory cytokines and chemokines from the synovium cause focal bone erosions in RA (51, 52). The concentrations of cytokines, chemokines, and other signaling molecules in the serum are much lower than that in the synovial fluid of RA patients (53-56). Therefore the effect of inflammatory cytokines, chemokines, and signaling factors present in serum of patients with active RA on bone mass might not be as potent as the impact of the inflammatory factors present in synovial fluid . Nevertheless, the complex combination of inflammatory cytokines, chemokines, and signaling factors present in the circulation of patients with active RA, might be sufficient to locally trigger inflammatory cytokine production within the bone in a positive feedback loop. Although the effects of individual recombinant cytokines on bone cell formation and function *in vitro* have been documented (10, 11, 27, 31, 32), the effect of

chemokines on bone cell function and communication is still unclear. Moreover, the effect of the complex combination of cytokines, chemokines, and signaling factors present in active RA-serum on bone cell formation, function, and communication has not yet been investigated. Studies on this subject are important since the effect of a combination of cytokines, chemokines, and other signaling factors might be different from the effect of each of these individual factors, which resembles more closely the *in vivo* situation.

Therefore, the main three aims of this thesis were: 1) to investigate whether active RA-serum containing a complex combination of inflammatory cytokines and signaling factors affects bone cell differentiation, function, and communication *in vitro*, 2) to investigate whether mechanical stimuli can reverse the effect of active RA-serum on osteocyte-to-osteoclast communication *in vitro*, and 3) to investigate whether the chemokines CXCL8 and CCL20 affect bone cell formation, function, and communication *in vitro*. This study is designed to improve our understanding of how systemic inflammation during RA plays a role in the emergence of generalized bone loss. Such insight also provides an increased understanding of the mechanism of bone loss during inflammation in general, which might contribute to the development of new therapeutic targets and interventions to prevent inflammation-induced bone loss. In order to achieve our aims, we addressed the following scientific questions:

1. Which *in vitro* research methods are available to explore the mechanisms of bone cell formation, function, and communication (**Chapter 2**)?
2. Does serum from patients with active RA affect differentiation of osteo/chondrogenic precursor cells (**Chapter 3**)?
3. Does serum from patients with active RA affect osteoblast proliferation, differentiation, and osteoblast-to-osteoclast communication (**Chapter 4**)?
4. Do chemokines affect osteoblast proliferation, differentiation, and osteoblast-to-osteoclast communication (**Chapter 5**)?
5. Does mechanical loading reverse the stimulatory effect of RA-serum on osteocyte-to-osteoclast communication (**Chapter 6**)?
6. Do exogenous recombinant inflammatory cytokines and serum from patients with active RA affect human osteocyte signaling (**Chapter 7**)?

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CHAPTER 2

Biomechanics of Bone Cells

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ABSTRACT

Osteocytes *in vivo* respond to mechanical stimuli with an altered production of growth factors. These growth factors modulate the behavior of bone resorbing cells, osteoclasts, and bone forming cells, osteoblasts, resulting in an adaptation of bone mass and structure. In this chapter we describe several *in vitro* methods for studying the communication between mechanosensitive bone cells, the osteocytes, with the effector cells, the osteoclasts and osteoblasts.

KEY WORDS:
Osteoblast, osteocyte, osteoclast, mechanical loading, bone formation, bone resorption

INTRODUCTION

Mechanosensitive bone cells translate mechanical stimuli into a biological response (1). The bone cell response to mechanical stimuli varies from one individual to another depending on age, sex, as well as physiological and pathological conditions (2). In the absence of mechanical stimuli, such as during disuse, stasis of interstitial fluid in bone occurs leading to a lack of fluid shear stress on the osteocytes (3). Osteocytes produce pro-osteoclastogenic signals in the absence of mechanical loading, leading to a stimulation of bone resorption (4). In the presence of mechanical stimuli, osteocytes produce factors that inhibit osteoclastogenesis, and/or decrease the production of osteoclast-stimulating signals (5). The most well known soluble factors affecting osteoclastogenesis are receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony-stimulating factor (MCSF), which stimulate osteoclast formation, and osteoprotegerin (OPG), which inhibits osteoclast formation. Under disuse conditions osteocytes produce RANKL, MCSF and OPG. In response to mechanical stimulation osteocytes produce factors (e.g. matrix extracellular phosphoglycoprotein; MEPE) which decrease RANKL, and increase OPG production (6) (Figure 1).

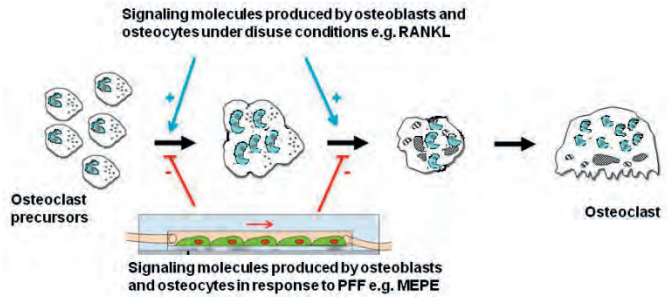

Figure 1. Biomechanical stimulation of osteocytes affects osteoclastogenesis and osteoclast activity. PFF, pulsating fluid flow.

To study the communication between mechanically stimulated osteocytes and osteoclast precursors *in vitro*, one requires a source of osteocyte-like cells, a method for mechanical stimulation, and a source of osteoclast precursors. Mechanical stimuli can be applied to osteocytes *in vitro* through various methods, such as pulsating fluid flow (PFF) (7). The protocol for performing PFF is described in chapter 37 of Experimental Research Methods. Osteocyte-like cells of human origin can be obtained by culturing human primary bone cells as outgrowth from long bones. Since osteocytes are terminally differentiated cells of the osteoblast lineage, mechanosensitive human osteoblast cell lines can be used as a model for

osteocytes. In studies where it is appropriate to use cells of animal origin, the mouse osteocyte cell line MLO-Y4 can be used or osteocytes can be isolated from the calvariae of chickens. Basic principles of culturing bone cells are described in chapter 35 of Experimental Research Methods. Bone marrow and peripheral blood mononuclear cells (PBMCs) are an excellent source of osteoclast precursors. PBMCs can be easily isolated from human peripheral blood, and when cultured with RANKL and MCSF these cells fuse and form multinucleated osteoclasts (8, 9). The formation of osteoclasts *in vitro* can be quantified relatively easily as osteoclasts are multinucleated and produce high levels of the enzyme tartrate-resistant acid phosphatase (TRAcP), which can be visualized by a TRAcP staining (9). In addition, PBMCs cultured on a bone or dentin slice become active, bone resorbing osteoclasts, and form a resorption pit on the slice. These resorption pits can be visualized by a Coomassie Brilliant Blue staining after removal of the cells (9-11) (Figure 2).

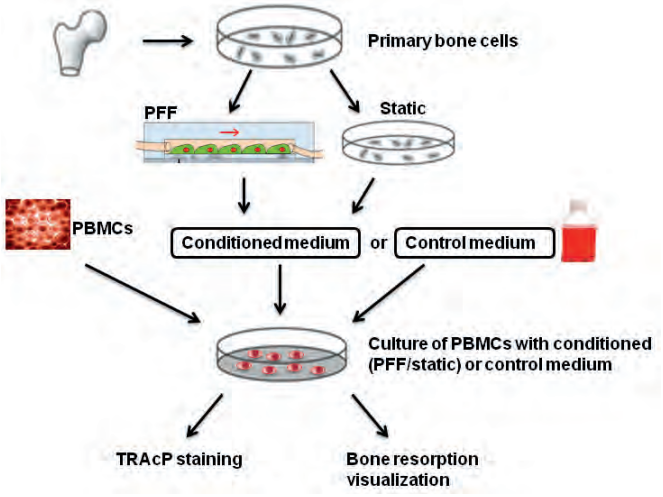

Figure 2. Experimental setup for studying the effect of biomechanical stimulation of osteocytes on osteoclastogenesis and osteoclast activity *in vitro*: Conditioned medium is collected from pulsating fluid flow (PFF) or statically cultured primary bone cells. Peripheral blood mononuclear cells (PBMCs) are cultured with conditioned medium or control medium. The effect of conditioned medium on osteoclast formation and osteoclast activity is analyzed by tartrate-resistant acid phosphatase (TRAcP) staining and the resorption pit assay respectively.

Culture medium taken from PFF-stimulated or statically cultured osteocytes (conditioned medium) contains growth factors produced by the osteocytes. Control medium (medium which has not been in contact with osteocytes) lacks osteocyte-growth factors. Compared to control medium, culturing

osteoclast precursors with static conditioned medium enhances osteoclast formation, while culturing osteoclast precursors with conditioned medium of PFF-stimulated osteocytes reduces osteoclast formation (5, 6).

In the first half of this chapter, we focus on *in vitro* methods that can be used to analyze the effect of conditioned medium of osteocytes on osteoclast formation and osteoclast activity. In the second half of this the chapter, we focus on *in vitro* methods for analyzing the effect of conditioned medium on proliferation and osteogenic differentiation of mesenchymal stem cells (MSCs). Under physiological conditions, osteocytes produce signaling factors that enhance osteogenic differentiation of stem cells (e.g. bone morphogenic proteins and Wnts) in response to mechanical loading (12) (Figure 3). MSCs are the progenitors of bone forming cells. They are often derived from bone marrow or adipose tissue for *in vitro* experiments.

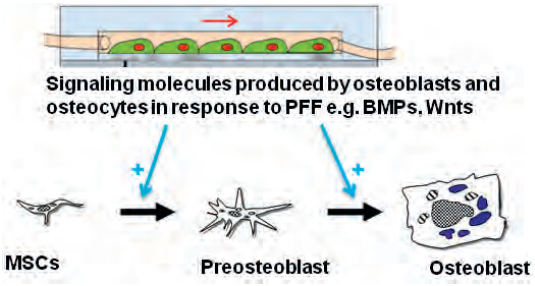

Figure 3. Biomechanical stimulation of osteocytes affects the differrentiation of mesenchymal stem cells (MSCs) towards osteogenic cells. BMP, bone morphogenic protein: PFF, pulsating fluid flow.

In this chapter we describe a protocol for the culture of human bone marrow-derived MSCs with conditioned medium of osteocytes or control medium, and the subsequent analysis of proliferation and osteogenic differentiation of the MSCs. Cell proliferation is estimated by measuring the cell number via quantification of DNA content. Osteogenic differentiation of MSCs can be determined by quantifying gene expression of osteogenic markers via PCR (see chapter 39 of Experimental Research Methods). Mature, active osteoblasts deposit mineralizing matrix in culture, which can be visualized by alizarin red or Von Kossa-staining. Osteocalcin and serum procollagen type 1 amino-terminal propeptide (P1NP) are released in the culture medium and can be analyzed with enzyme-linked immunosorbent assay (ELISA) (Figure 4).

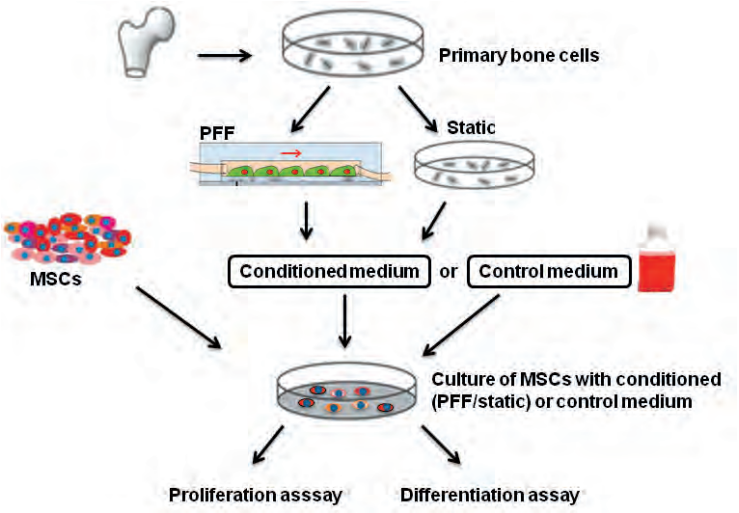

Figure 4. Experimental setup for studying the effect of the biomechanical stimulation of osteocytes on MSCs differentiation towards osteogenic cells *in vitro*: Conditioned medium is collected from pulsating fluid flow (PFF) or statically cultured primary human bone cells. Mesenchymal stem cells (MSCs) are cultured with conditioned medium or control medium. The effect of conditioned medium on MSCs proliferation is analyzed by deoxyribonucleic acid (DNA) quantification. The effect of conditioned medium on osteogenic differentiation of MSCs is analyzed by measuring ALP activity, osteogenic gene expression (e.g. *OCN, OPN, RUNX2, DMP1*) and mineral deposition and matrix formation (Alizarin red staining and quantification).

MATERIALS AND REAGENTS

All reagents, materials, and culture medium used for cell purification and cell culture should be sterile.

OSTEOCLASTOGENESIS AND OSTEOCLAST ACTIVITY ASSAYS

Osteoclast precursors (PBMCs) isolation

1. Buffy coat (obtained form Blood bank) or venipuncture blood with anticoagulant (e.g. EDTA or heparin)
2. 1% Phosphate-buffered saline-citrate (PBS-citrate): mix 10 ml citrate stock in 1000 ml PBS at room temperature (RT), and put the bottle on ice until use.
   Citrate stock: dissolve 456 g of sodiumcitrate-dihydrate (MW=294) and 21.4 g of citrate-monohydrate (MW=192.12) up to 1 liter of Milli-Q water and autoclave.
3. Lymphoprep (Ficoll)
4. 50 ml tubes
5. Pasteur pipettes
6. Cell culture flasks (T75) and 96-well plates

PBMCs culture

1. Dulbecco's modified Eagle's medium (DMEM)
2. Fetal clone serum (FCS) (see Note 1)
3. Penicillin-Streptomycin-Fungizone; PSF (Sigma #A-5955)
4. MCSF (R&D Systems #216-MC)
5. RANK-L (Peprotech #310-01)
6. Conditioned medium: Collect the conditioned medium after 60 min of PFF/static culture of primary human bone cells and store the medium at -20°C (see Note 2)
7. Control medium: DMEM + 10% FCS + 1% PSF + MCSF (50 ng/ml) + RANKL (80 ng/ml). Make fresh control medium each time
8. 96-well culture plates (Greiner # 655180) (see Note 3)

TRAcP staining

1. Leukocyte acid phosphatase TRAcP kit (Sigma # 387A- 1 kt)
2. PBS-buffered 4% formaldehyde
3. Milli-Q water
4. Potassium sodium tartrate solution (1 mol/l); dissolve 2.8 g in 10 ml Milli-Q water
5. 4', 6-diamidino-2-phenylindole (DAPI), stock = 100 mg/ml in PBS, dilute 100x to make a working solution (see Note 4)

Bone resorption visualization

1. Bovine cortical bone slices (thickness 0.5 mm)
2. PBS
3. 70% ethanol
5. Medium: DMEM + 10% FCS + 1% PSF
4. Forceps
5. 10% ammonium hydroxide (NH_4OH) solution
6. Approximately 10% water saturated alum [$KAl(SO_4)_2*12H_2O$]. Filter before use
7. Coomassie brilliant blue (CBB; PhastGel Blue R, Pharmacia, Uppsala, Sweden). Dissolve 1 tablet in 80 ml water for 5 min and add 120 ml methanol. The solution is stable for a couple of months in the refrigerator. Dilute 1:1 in 20% acetic acid directly before use. Filter this solution (Wattman) before use

PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF MSCS

MSCs culture

1. Human MSCs, bone marrow (Lonza Cologne GmbH)
2. Dulbecco's modified Eagle's medium (DMEM)
3. Fetal clone serum (FCS)
4. Penicillin-Streptomycin-Fungizone; PSF (Sigma #A-5955)
5. 20 mM β-glycerophosphate (Sigma); dissolve 14.79 g/ 50 ml PBS (1 M), heat at 50ºC for 10 min to dissolve. Filter and divide in 1 ml aliquots, and store at -20 ºC. Dissolve in 50 ml medium to make a final concentration of 20 mM
6. 100 µM ascorbic acid (Sigma); dissolve at 5 mg/ml PBS, filter, and add 1 ml to 50 ml medium to make a final concentration of 100 µM (make fresh solution before each use)
7. 48-well culture plates

DNA quantification

1. PBS
2. Milli-Q water
3. CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen)

Alkaline phosphatase activity

1. ALP IFCC liquid assay (Roche)
2. Protein assay (BCA; BioRad)

Analysis of osteogenic gene expression

1. RNA isolation spin columns (e.g. QIAGEN-RNeasy)
2. Random primers designed in Primer Blast software
3. Reverse transcriptase kit (e.g. SuperScript® VILO™ cDNA Synthesis Kit)
4. Primers and master mix for SYBR green (e.g. SYBR® Green Supermix BioRad)
5. Light Cycler system to quantify amplified cDNA (e.g. Light Cycler® 480)

Alizarin red-staining

1. 4% formaldehyde
2. 40 mM alizarin red S; dissolve 500 mg of alizarin red in 40 ml dH_2O. Adjust pH to 4.1 with 0.5% ammonium hydroxide

Alizarin red-staining quantification

1. 10% acetic acid
2. Cell scraper
3. Parafilm or mineral oil
4. 0.5% ammonium hydroxide
5. Opaque-walled, transparent-bottom 96-well plates

METHODS

Osteoclastogenesis and osteoclast activity

In short, preparations for this experiment are as follows: Obtain human trabecular bone (e.g. surgical waste material) and mince into small pieces. Incubate bone pieces at 37°C with collagenase type II (Worthington, NJ) for 2 h. After washing several times with PBS, transfer the bone pieces to a culture flask with DMEM+ 10% FCS + 1% PSF (13). Primary bone cells will grow out of the bone pieces and reach confluence within 3-4 weeks. Subject the primary human bone cells to PFF or static control culture and analyze the production of known signaling molecules (e.g. NO and PGE_2) as described in chapter 37 of Experimental Research Methods. Collect the conditioned medium and store as described under section "PBMCs culture" of this chapter.

Isolation of PBMCs from buffy coat or venipuncture

1. Prepare 1% PBS-citrate from the citrate stock at room temperature (RT).
2. Transfer the buffy coat to a T 75cm^2 flask (see Note 5) and dilute 1:1 with the 1% PBS-citrate (e.g. add 50 ml PBS-citrate solution to 50 ml buffy coat). Mix very gently (do not shake).
3. Fill four 50-ml tubes with 15 ml of Lymphoprep (Ficoll) per tube at RT.
4. Layer the buffy coat on the Lymphoprep (Ficoll) in the tubes. Do this carefully, drop by drop, with a 25 ml pipette. Do not mix the cells with the lymphoprep (most tricky step). Layer 2 tubes with 25 ml buffy coat and 2 tubes with 20 ml buffy coat. Discard the remaining buffy coat.
5. Centrifuge 4 tubes for 30 min at 800*x*g at room temperature. Do not use brakes to stop the centrifuge.
6. Carefully take off the interphase containing monocytes and lymphocytes with a pasteur pipette and transfer to two 50-ml tubes.
7. Fill the tubes with 1% PBS-citrate up to 50 ml.
8. Centrifuge for 10 min at 400*x*g at RT.
9. Discard the supernatant carefully so you will not discard the cells.
10. Very gently resuspend the cell pellet of bottom of each tube in 5 ml 1% PBS-citrate at RT, and fill with 1% PBS-citrate (4ºC) up to 50 ml (mix gently). Never shake your tubes as this will activate the monocytes and lower the yield.
11. Centrifuge the tubes for 5 min at 400*x*g at 4ºC.
12. Repeat steps 9-11 three times, until the supernatant is completely clear (colorless).

Culture of osteoclast precursors with conditioned medium and control medium

1. Dilute the PBMCs obtained from section “Isolation of PBMCs from buffy coat or venipuncture” to $1.3x10^7$ cells/ml with control culture medium.
2. Pipette 75 µl cell suspension per well in a 96-well plate. Add 75 µl PFF-conditioned medium or 75 µl static-conditioned medium or 75 µl DMEM culture medium without serum. Final cell density: 1×10^6 cells/well; final MCSF concentration: 25 ng/ml; final RANKL concentration: 40 ng/ml; final volume per well in 96-well plate: 150 µl).
3. Gently refresh the medium with 1:1 diluted medium every three days in consecutive wells: 150 µl conditioned medium (diluted with control medium) and 150 µl control medium (diluted with DMEM).
4. Continue to culture for 3 weeks.

Visualization of osteoclasts by TRAcP staining

1. Wash the cells with PBS and fix the cells with PBS-buffered 4% formaldehyde.
2. Wash the cells with 37°C Milli-Q water.
3. Mix 25 µl of fast garnet GBC base and 25 µl of sodium nitrate solution from the kit.
4. Prepare mix solution:

Milli-Q water at 37°C	4.5 ml
Solution from point 3	50 µl
Napthol AS-BI solution	50 µl
Acetate solution	200 µl
Tartrate solution, 1 M	250 µl

5. Cover the cells with mix solution (50 µl in a 96-well plate). Incubate for 30 min at room temperature in the dark.
6. Wash the cells gently with tap water and stain the nuclei with DAPI for 2 min.
7. Wash the cells gently with tap water at RT. TRAcP positive cells stain dark red with light blue nuclei (DAPI) as shown in Figure 5.
8. Categorize TRAcP positive cells: mononuclear cells and multinuclear cells. Sub categorize the multinucleated TRAcP positive cells; 3 nuclei, 3-6 nuclei, >6 nuclei.
9. Count the number of TRAcP positive cells/per well according to categories using a microscope with 20x objective.

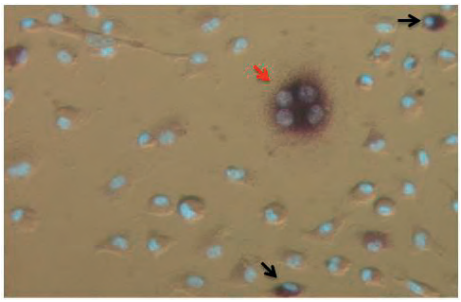

Figure 5. TRAcP positive multinucleated cell (i.e. osteoclast, red arrow) and TRAcP positive mononuclear cells (black arrows).

Analysis of osteoclast activity by resorption pit assay

1. Wash the bone slices (stored in 70% ethanol) several (at least 3) times with PBS before use. Put the bone slices in a 96-well plate with sterile forceps, add the medium, and put for at least half an hour in the incubator.
2. Seed the osteoclast precursor cells and replace the media (as described under section “Culture of osteoclast precursors with conditioned medium and control medium” of this chapter).
3. After 3 weeks wash the bone slices with water. If necessary the slices can be stored after this step for up to one month in the refrigerator.
4. Pipette the water from the bone slices and sonicate for 30 min in 10% NH_3OH on ice.
5. Wash the bone slices twice with water in the wells in a fume cabinet (ammonium hydroxide is toxic), and bring back to the lab bench. Dry bone slices on a filter paper, but do not allow them to dry completely. Store the slices in tap water if you have a large series of experiments.
6. Transfer the bone slices to a new well of a 96-well plate, wash them with a small volume of water-saturated alum, and subsequently incubate for 10 min in fresh water. Make sure that both sides of the bone chip have been covered by the alum by placing the bone chips vertically in the well.
7. Wash the slices thoroughly again, twice with tap water. Leave the bone slices in tap water. Pick up a bone slice, one-by-one, and splash once with a strong spurt (water siphon).
8. Completely dry both sides of the bone slices between filter paper. Pick up the bone slices, one-by-one, and splash once with a strong water spurt rinse of CBB solution to both sides of the bone slice using a pasteur pipette. Decant excess of CBB on the side of the tube and dry immediately between filter paper, press on it. Transfer to a clean well in a well plate. Bone resorption is visible as blue resorption pits (Figure 6); visualize the pits using a light microscope.

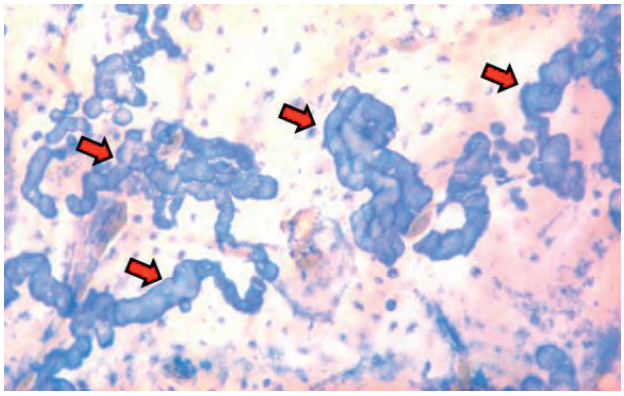

Figure 6. Resorption pits (blue area indicated by arrows) made by an active osteoclast on the bone surface visualized by light microscopy.

Proliferation and osteogenic differentiation of MSCs

Mesenchymal stem cells culture

1. Prepare osteogenic medium: DMEM + 10% FCS + 1% PSF + 20 mM β-glycerophosphate + 100 µM ascorbic acid (see Note 6).
2. Dilute the MSCs (see Note 7) to $4x10^4$ cells/ml with osteogenic medium.
3. Pipette 125 µl cell suspension per well in 48-well plates. Add 125 µl PFF-conditioned medium, static-conditioned medium, and osteogenic-conditioned medium in consecutive wells. So, final cell density: $5x10^3$ cells/well; final β-glycerophosphate concentration: 10 mM; final ascorbic acid concentration: 50 µM; final volume in 48-well plate: 250 µl.
4. Refresh the media with 1:1 diluted media every 3 days in consecutive wells; 250 µl conditioned medium (diluted with osteogenic medium) and 250 µl osteogenic medium (diluted with DMEM).
5. Measure DNA content on day 5, 7, 10, and 14.
6. Measure ALP activity on day 5, 7, 14, 21, and 28.
7. Isolate total RNA on day 5, 7, 14, 21, and 28.
8. Perform alizarin red-staining on day 14, 21, and 28 because osteogenic cells produce mineralizing matrix at these late time points.

DNA quantification

1. Wash the cells with PBS.
2. Lyse the cells with 0.5 ml ice cold Milli-Q water and collect the lysate.
3. Sonicate the lysate 2x for 30 sec.
4. Centrifuge for 5 min at 5000 rpm, collect the supernatant, analyze directly or stored at -20°C.

5. Analyze the total DNA content from the supernatant according to the manufacturer's protocol; CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen) (see Note 8).

Alkaline Phosphatase activity

1. Take the supernatant of the lysate obtained from section "DNA quantification"
   of this chapter. Avoid freeze-thaw cycles.
2. Analyze ALP activity from the cell lysate using the ALP IFCC liquid assay (Roche) kit according to the manufacturer's instruction.
3. Analyze total protein from the cell lysate using the BioRad protein assay according to the manufacturer's instruction.
4. Calculate ALP activity per amount of protein.

Analysis of osteogenic gene expression

1. Isolate total RNA using commercially available RNA isolation spin columns kit.
2. Synthesize cDNA from total RNA using random primers as described in chapter 39 of Experimental Research Methods.
3. Analyze osteogenic gene expression by using specific primers listed in Table 1, SYBR® Green Supermix in the LightCycler® 480. Commonly used housekeeping genes (see Note 9) are glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), TATA-box binding protein (*TBP*), and tyrosin-monooxygenase activation protein (*YWHAZ*).

Alizarin red-staining

1. Carefully aspirate the medium from each well. Be careful to not aspirate the cells.
2. Wash cells once with 2 ml PBS.
3. Fixate cells by covering with 4% formaldehyde and incubating at RT for 15 min.
4. Carefully remove the fixative and rinse cells three times (5-10 min each) with Milli-Q water. Wash gently to avoid disturbance of the monolayer.
5. Remove water and add 0.5 ml/well alizarin red-staining solution. (Von Kαossa-staining can also be used instead of alizarin red-staining).
6. Incubate at RT for at least 20 min.
7. Wash 4 times with Milli-Q water by gently shaking for 5 min to remove excess dye.
8. Add 0.5-1 ml water to each well to prevent the cells from drying. The plate is now ready for visual inspection and/or image acquisition under light microscope. Cells that have differentiated into osteoblasts (showing mineral

formation and matrix deposition) produce bright red nodules as shown in Figure 7.

Table 1. Primer sequences for determination of osteogenic differentiation through PCR.

Gene		Oligonucleotide sequence	Product Size (bp)
ALP	Forward	5' GCTTCAAACCGAGATACAAGCA 3'	102
	Reverse	5'GCTCGAAGAGACCCAATAGGTAGT 3'	
OPN	Forward	5' TTGCTTTTGCCTCCTAGGCA 3'	430
	Reverse	5' GTGAAAACTTCGGTTGCTGG 3'	
OCN	Forward	5' GCTACCTGTATCAATGGCTG 3'	222
	Reverse	5' GGAAGAGGAAAGAAGGGTG 3'	
RUNX2	Forward	5' ATGCTTCATCGCCTCAC 3'	165
	Reverse	5' ACTGCTTGCAGCCTTAAAT 3'	
DMP-1	Forward	5' AGCATCCTGCTCATGTTCCTTT 3'	103
	Reverse	5' CCAAATGACCCTTCCATTCTTC 3'	
MEPE	Forward	5' GAGTTTTCTGTGTGGGACTACTCT 3'	102
	Reverse	5' TCTGCTCTTCCACACAGCTTTG 3'	
GAPDH	Forward	5' ATGGGGAAGGTGAAGGTCG 3'	70
	Reverse	5' TAAAAGCAGCCCTGGTGACC 3'	

ALP: Alkaline phosphatase; *OPN*: Osteopontin; *OCN*: Osteocalcin; *RUNX2*: Core binding factor α 1, also known as *CBFA1*; *DMP1*: Dentin matrix acidic phosphoprotein 1; *MEPE*: Matrix extracellular phosphoglyco-protein; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

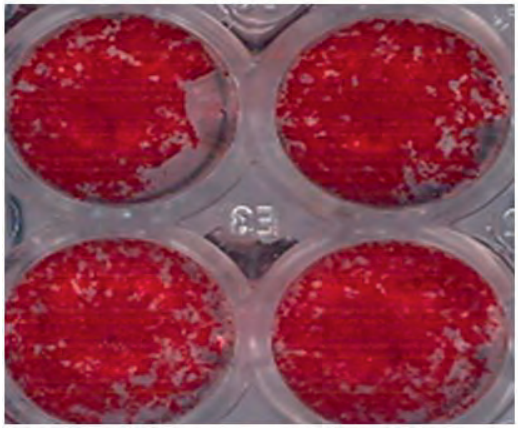

Figure 7. Alizarin red-staining of mineralized matrix formed by osteoblasts.

Quantification of alizarin red-stained mineral and matrix

1. Add 200 µl of 10% acetic acid to each alizarin red-stained well of a 48-well plate and incubate for 30 min under gentle shaking.
2. The cell monolayer will now be loosely attached. Gently scrape the cells from the plate and transfer cells and acetic acid to a 1.5 ml microcentrifuge tube.
3. Vortex vigorously for 30 sec.
4. Heat to 85°C for 10 min. To avoid evaporation, seal the microcentrifuge tube with parafilm or overlay the sample with 200 µl mineral oil.
5. Transfer the tube to ice for 5 min. Do not open the tube until fully cooled.
6. Centrifuge the slurry at 20,000*x*g for 15 min.
7. Prepare alizarin red-standard serial dilutions from alizarin red-solution with Milli-Q water (2 mM, 1 mM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.3 µM, and blank). You can change standard dilutions according to cells staining intensity.
8. After centrifugation transfer 200 µl of the supernatant to a new 1.5 ml microcentrifuge tube.
9. Neutralize the pH with ~75 µl of 0.5% ammonium hydroxide (sample/standard). Take a small aliquot and test the pH to ensure that it is in the range of 4.1-4.5.
10. Pipette 100 µl of the standard/sample (25 µl sample + 75 µl Milli-Q water) to an opaque-walled, transparent bottom 96-well plate.
11. Measure the optical density (OD) at 405 nm.
12. Plot the standard curve according to concentration *vs.* OD of the standards.
13. Quantify the alizarin red-stained mineral and matrix according to standard curve and OD of your samples.

CONCLUSION

Studies on the response of osteocytes to mechanical stimuli are highly important in order to gain insight in how bone homeostasis is disturbed in diseases such as rheumatoid arthritis, osteoarthritis, or osteoporosis. In chapter 37 of this book, the protocol for subjecting mechanosensitive bone cells to mechanical stimuli *in vitro* has been described in detail. In the current chapter a protocol is provided that allows investigation of the effect of mechanical stimulation on the production of factors by osteocyte-like cells that affect bone resorption and bone formation. These *in vitro* experiments may help clinicians and researchers to unravel how biomechanics of bone cells varies from one individual to another depending on age, sex, and physiological and/or pathological conditions, and how biomechanics of bone cells affects bone homeostasis.

NOTES

1. The exact constitution of serum batches can vary strongly, thereby influencing the outcome of cell culture experiments. It is therefore of utmost importance to test several serum batches (e.g. culture PBMCs with different batches and measure the effect on osteoclast formation), choose the batch that performs best, and use the same batch of serum during experiments.
2. The signaling molecules produced by bone cells on the response to PFF will generally be released within 60 min after the start of PFF. In order to study the effect of signaling molecules produced *de novo* by osteocytes in response to PFF, or to concentrate the amount of proteins in the conditioned medium, continue the cell cultures for 2-48 h after PFF before collecting the conditioned medium.
3. The 96-well plates have a high clarity for optimal microscopic examination, and have undergone a physical surface treatment that improves cell adhesion.
4. Potassium sodium tartrate and DAPI are toxic. Use gloves and take general safety measures during the use of these chemicals.
5. Osteoclast precursors can be obtained from peripheral blood, but also from bone marrow. Due to the invasive medical procedure needed to get human bone marrow and the low amount of bone marrow that can be obtained, PBMCs are a preferable source of human osteoclast precursors for *in vitro* experiments.
6. Platelet lysate enhances osteogenic differentiation of MSCs better and faster than FCS, therefore 5% platelet lysate can be used instead of 10% FCS.
7. Bone marrow is not the only source of MSCs. Adipose tissue obtained by liposuction is also a good source of MCSs and is often available in relatively large quantities.
8. The DNA content in the cell lysate is directly proportional to cell number. DNA quantification can thus serve as an estimate of cell proliferation. An alternative method for determining cell number is the XTT assay (XTT proliferation kit II; Roche). In addition, one can measure markers for proliferation during PCR, such as Ki67 and PCNA.
9. Housekeeping genes are essential for basic cellular function. These genes are expressed at relatively constant level in all cells of an organism under normal and patho-physiological conditions. To measure the effect of a stimulus (e.g. BMPs, Wnts) on target gene expression (e.g. ALP, OCN) that target gene is divided by housekeeping gene expression as an indirect correction for cell number.

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CHAPTER 3

Serum of Patients with Active Rheumatoid Arthritis Inhibits Differentiation of Osteochondrogenic Precursor Cells

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ABSTRACT

Delayed fracture healing is frequently experienced in patients with systemic inflammation such as during rheumatoid arthritis (RA). The reasons for this are diverse, but could also be caused by inflammatory cytokines and/or growth factors in serum from patients with active disease. We hypothesized that serum from patients with active RA contains circulating inflammatory factors that inhibit differentiation of osteochondrogenic precursors. Serum was obtained from 15 patients with active RA (active RA-sera) and from the same patients in clinical remission 1 year later (remission RA-sera; controls). The effect of active RA-sera on osteochondrogenic differentiation of chondrogenic ATDC5 cells and primary human periosteum-derived progenitor cells was determined in micromass culture. In ATDC5 cells, active RA-sera reduced *Ki67* transcription levels by 40% and cartilage matrix accumulation by 14% at day 14, and *Alp* transcription levels by 16%, and matrix mineralization by 17% at day 21 compared to remission RA-sera. In human periosteum-derived progenitor cells, active RA-sera inhibited metabolic activity by 8%, *SOX9* transcription levels by 14%, and cartilage matrix accumulation by 7% at day 7 compared to remission RA-sera. In conclusion, sera from patients with active RA negatively affect differentiation of osteochondrogenic precursors, and as a consequence may contribute to delayed fracture healing in these patients.

KEY WORDS:
Rheumatoid arthritis, systemic inflammation, inflammatory cytokines, osteochondrogenic differentiation, fracture healing

INTRODUCTION

Systemic inflammation during inflammatory diseases such as rheumatoid arthritis (RA) is closely associated with bone loss and generalized osteoporosis (1, 2). This leads to increased fracture risk, and is therefore included in the fracture risk assessment tool (FRAX) (3). Complications in bone healing including delayed fracture healing are experienced during systemic inflammation (2, 4-6). Therapeutic agents such as cyclooxygenase (COX) inhibitors and corticosteroids are factors involved in delayed fracture healing and could partly explain the delayed fracture healing observed in patients with chronic inflammation (7-9). Mechanical loading enhances endochondral ossification and fracture healing (10). Patients with RA show limited physical activity, and if a fracture occurs it causes even more limited physical activity, which might be the cause of the delayed fracture healing observed in these patients (11). Proper homing of progenitor cells is essential for fracture healing, and localized inflammation at the fracture site triggers the homing of these cells (12, 13). In RA, systemic inflammation might negatively affect the acute inflammation-mediated precursor cell homing thereby causing delayed fracture healing. In addition, RA-sera have been shown to directly inhibit osteoblast differentiation and enhance osteoblast-mediated osteoclastogenesis (14). Therefore the factors in the circulation of patients with active RA might delay fracture healing by directly affecting osteochondrogenic differentiation of precursor cells.

Endochondral ossification starts with chondrogenic differentiation of precursor cells (15, 16). Cartilaginous nodules formed during chondrogenesis are rich in glycosaminoglycan (GAG) content, and coincide with the up-regulation of the key chondrogenic marker SOX9, which decreases as the conversion to hypertrophy begins. At the same time, upregulation of the hypertrophic marker COL10A, and the osteogenic markers RUNX2 and ALP occurs (15, 16). Periosteum-derived mesenchymal stem cells are progenitor cells contributing to endochondral ossification during fracture healing (17). Osteochondrogenic differentiation of periosteum-derived cells is essential for the process of fracture healing which recapitulates intramembranous and endochondral bone formation (18). Osteochondrogenic differentiation of progenitor cells during bone healing is triggered by multiple molecular signals that are released during the initial inflammatory stage of fracture repair (19, 20). However chronic systemic inflammation introduces complications during fracture healing and causes delayed fracture healing (2).

Cells in inflamed synovia in RA produce high amounts of proinflammatroy cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-17 (IL-17), tumor necrosis factor-α (TNFα), and CXCL8 (IL-8) (21, 22). Elevated levels of these cytokines are found in synovial fluid and serum from RA patients (23, 24). IL-

1β and TNFα cause restricted recovery of growth plate chondrogenesis and longitudinal bone growth in murine postnatal metatarsal bones (25). Continuous long-term IL-6 treatment and late short-term IL-1β, TNFα, or IL-6 treatment inhibit chondrocyte differentiation and function (26, 27). IL-6 negatively regulates osteoblast differentiation (28). Early short term TNFα treatment on the other hand enhances, while continuous TNFα treatment inhibits osteogenic differentiation of mesenchymal stem cells (29). However the effect of continuous treatment with inflammatory mediators present in active RA-sera on osteochondrogenic differentiation still needs to be unraveled.

We hypothesized that one of the mechanisms leading to delayed fracture healing in RA is inhibition of osteochondrogenic differentiation of precursor cells by circulating inflammatory factors as present in the active RA-serum. In the current study, we cultured the murine chondrogenic ATDC5 cell line and primary human periosteum-derived progenitor cells (HPDC) with serum obtained from 15 well characterized patients with active early RA before starting disease-modifying anti-rheumatic drugs (DMARDs) therapy. Serum obtained from the same 15 patients during clinical remission 1 year later were used as control for each active RA-serum. We analyzed the effect of active RA-sera on osteochondrogenic differentiation of ATDC5 cells and HPDC in micromass culture.

MATERIALS AND METHODS

Selection of RA patients and serum collection

Fifteen RA patients were recruited from the CareRA project coordinated by the clinical rheumatology team of the Skeletal Biology and Engineering Research Center, KU Leuven, Belgium (mean age: 60±14 yrs; 12 females, 3 males). In CareRA patients with RA according to the 1987 classification criteria were recruited at an early stage of the disease (<1 yr disease duration) and *before* they had taken DMARDs or corticosteroids. Blood samples were collected, and within 1 h these were centrifuged for 10 min at 3000 rpm to separate the sera, which were aliquoted and stored at −80ºC. Patient characteristics, demographics, and clinical data (anti-citrullinated protein antibodies, rheumatoid factor, and erosions) were collected at baseline (Table 1). Then standardized treatment was started with methotrexate (MTX) or a combination of MTX with sulphasalazine or leflunomide and corticosteroids for 34 weeks. After 34 weeks the corticosteroids were stopped, and in those patients that had also received DMARDs at baseline, DMARD treatment was stepped down to a monotherapy with MTX from week 40 onwards. At 52 weeks, blood samples were collected once again. Sera were separated from the blood samples, and stored as described above. Patients with thyroid dysfunction, other inflammatory diseases than RA, and pregnancy were not included in this study. Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and DAS28CRP were measured at baseline when patients had active untreated disease and after 52 weeks when disease activity was controlled with medication (Table 2). In addition, a healthy control sera pool was made by pooling the sera from 22 healthy donors (age: 41±18 yrs; 14 females, 8 males). The serum CRP level of the individual healthy control sera, as well as the healthy control pool was <2.5 mg/l, which indicates that none of the healthy donors suffered from inflammatory disease during the time of serum collection. This study was approved by the local ethical committee of the Leuven University Hospital, Belgium, and the Ethical Review Board of the VU University Medical Center, Amsterdam, The Netherlands. All subjects gave informed consent.

Table 1. Characteristics and demographics of the 15 patients included in this study

	active RA (n=5) for ATDC5 culture	active RA (n=10) for HPDC culture
Sex (female/male)	4/1	8/2
Age, yrs	55.6 ± 18.4	60.7 ± 2.4
Weight, kg	63.1 ± 16.8	57.3 ± 6.4
BMI, kg/m2	25.1 ± 3.8	24.7 ± 3.0
# anti-CCP positive patients	2	7
# RF-positive patients	2	3
# patients with erosions	0	5
# patients with NSAIDs medication	5	8

Values are mean±SD. BMI, body mass index; anti-CCP, anti-citrullinated protein antibodies; RF, rheumatoid factor; NSAIDs, non-steroidal anti-inflammatory drugs.

Table 2. Clinical data of the patients included in this study at baseline and at 52 weeks

	baseline	52 weeks	p value
For ATDC5 (n=5)			
DAS28 score	6.0 ± 0.9	1.9 ± 0.7	0.0002*****
CRP (mg/l)	40.3 ± 38.4	1.8 ± 2.0	0.039*****
ESR (mm/h)	64.2 ± 21.4	37.2 ± 33	0.041*****
For HPDC (n=10)			
DAS28 score	5.0 ± 1.0	1.5 ± 0.5	<0.0001*****
CRP (mg/l)	11.1 ± 12	3.0 ± 3.7	0.036*****
ESR (mm/h)	29.5 ± 19	14.3 ± 9.1	0.027*****

Values are mean±SD. n, number of patients. *Significant effect of disease modifying anti-rheumatic drugs, p <0.05. DAS28, disease activity score; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

Micromass culture of ATDC5 cells with RA-sera

ATDC5 cells (Riken Cell Bank, Ibaraki, Japan) were expanded in a maintenance medium consisting of Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (DMEM/F12) (1:1 (vol/vol); Invitrogen, Carlsbad, CA), supplemented with 5% fetal bovine serum (FBS; Gibco, Grand Island, NY), 1% antibiotic–antimycotic solution (100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; Invitrogen), 10 µg/ml human transferrin (Sigma, St. Louis, MO),

and 30 nM sodium selenite (Sigma) as has been described earlier (30). Cells were grown in a humidified incubator at 37ºC and 5% CO_2 in air.

Subconfluent ATDC5 cells (passage 6) were released with trypsin (Gibco), centrifuged, and resuspended in maintenance medium at $2x10^7$ cells/ml. Ten µl of this cell suspension containing $2x10^5$ cells was pipetted in the center of each well of a 24 well plate (Greiner Bio-One, Kremsmuenster, Austria), and micromasses were allowed to form for 2 h. Then maintenance medium was added (0.5 ml/well) for 1 day, followed by a medium change with chondrogenic differentiation medium consisting of DMEM/F12 with 1x insulin-transferrin-selenium (ITS; Sigma), 100 µM ascorbic acid (Sigma), 100 nM dexamethasone (Sigma), 1% antibiotics, and 5 µg/ml human transferrin. The differentiation medium was also supplemented with either 10% active RA-serum or remission RA-serum. Micromasses were cultured up to day 21. Medium was refreshed every 3 days. For osteogenic differentiation and mineralization 7 mM β-glycerophosphate was added to the differentiation medium from day 14 to 21.

HPDC isolation and culture

Periosteal biopsies (0.5 cm^2) were harvested from the medial side of the proximal tibia (mean age 29±12 yrs, 2 females, 2 males) during total knee replacement surgery or distraction osteogenesis as described previously (31). Briefly, the periosteum was stripped from the tibia with a periosteal lifter. The periosteal specimens were transported in growth medium consisting of high-glucose DMEM supplemented with 10% FBS (BioWhittaker, Verviers, Belgium) and 1% antibiotic–antimycotic solution. The biopsies were finely minced and digested overnight in 0.2% type IV collagenase (Invitrogen) in growth medium at 37°C (as described above). Subsequently periosteal cells were collected by centrifugation, resuspended in growth medium and cultured in T25 flasks. After 5 days of culture non-adherent cells were removed by changing the medium. Upon reaching ~80% confluency the cells from 4 donors were harvested and pooled to reduce noise from genetic variability. These HPDC were used for further experiments. The ethical committee for Human Medical Research (Katholieke Universiteit Leuven) approved all procedures, and informed consent was obtained from all patients.

Micromass culture of HPDC with RA-serum

Subconfluent HPDC (passage 7) were released with trypsin (Gibco), centrifuged, and resuspended in growth medium at $2x10^7$ cells/ml. Ten µl of this cell suspension containing $2x10^5$ cells was pipetted in the center of each well of a 24 well plate (Greiner Bio-One), and micromasses were allowed to form for 2 h (32). Then growth medium was added (0.5 ml/well) for 1 day, followed by addition of chondrogenic differentiation media containing DMEM/F12 supplemented with 1x ITS, 100 µM ascorbic acid, 100 nM dexamethasone, 10 µM ROCK inhibitor

(Y27632; Axon Medchem BV, Groningen, The Netherlands), 40 µg/ml proline (Sigma), 1% antibiotic–antimycotic solution. The differentiation medium was also supplemented with either 10% active RA-serum or remission RA-serum or healthy control serum pool. Micromasses were cultured up to day 21. Medium was refreshed every 3 days. Metabolic activity of cells was analyzed at day 7 by using presto blue (Invitrogen) according to manufacturer's instruction. For osteogenic differentiation and mineralization 7 mM β-glycerophosphate was added to the differentiation medium from day 14 to 21.

Alcian blue and alizarin red staining

Accumulation of cartilage matrix in ATDC5 and HPDC micromass cultures was measured by alcian blue staining at day 14 by using 0.1% alcian blue 6x (Sigma) in 0.1 M HCl. For quantitative analysis, stained micromasses were dissolved with 6 M guanidine hydrochloride (Sigma) in water, and the absorbance was measured at 620 nm. Matrix mineralization was analyzed by alizarin red staining at day 21 by using 1% Alizarin Red S (Sigma) in water at pH 4.1. For quantitative analysis, stained micromasses were dissolved with 10% pure cetylpyridinium chloride (Sigma) in water, and absorbance was measured at 570 nm.

RNA isolation and real-time RT-PCR

Total RNA was isolated at day 14 and 21 from ATDC5 micromass cultures, and at day 7 and 21 from HPDC micromass cultures, by using an RNeasy® mini (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Total RNA concentrations were measured with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA synthesis was performed in a thermocycler GeneAmp® PCR System 9700 PE (Applied Biosystems, Foster City, CA), using a PrimeScript™ RT reagent Kit (Takara bio, Shiga, Japan) with 0.2 µg of total RNA in 10 µl reaction mixture consisting of TAKARA Reaction Mix and SuperScript Enzyme Mix. cDNA was stored at −20°C until real-time PCR analysis. Real-time PCR reactions for cDNA from ADTC5 cells were performed using 1 µl cDNA and SYBR® Premix Supermix (Takara bio) according to the manufacturer's instructions in a LightCycler®. For quantitative real-time PCR, the values of relative target gene expression were normalized for *Hprt* housekeeping gene expression. Real-time PCR was used to assess expression of the following genes: *Ki67,* collagen 2a1 (*Col2a1*), aggrecan (*Acan*), collagen 10a (*Col10a*), *Runx2*, alkaline phosphatase (*Alp*) and osteocalcin (*Ocn*). Real-time PCR reactions for cDNA from HPDC were performed using 1.0 µl cDNA and SYBR® Green Supermix (Roche Laboratories, Indianapolis, IN) according to the manufacturer's instructions in a LightCycler®. For quantitative real-time PCR, the values of relative target gene expression were normalized for *HPRT* housekeeping gene expression. Real-time PCR was used to assess expression of the following genes: *KI67*, collagen 2a1 (*COL2a1*), aggrecan

(*ACAN*), *SOX9*, *RUNX2, ALP* and osteocalcin (*OCN*). The primer sequences are listed in Table 3.

Statistical analysis

Gene expression data are expressed as median with 5-95 percentile range. The effects of active RA-sera on osteochondrogenic differentiation were tested using a two-tailed paired t-test. Differences were considered significant if $p<0.05$. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA).

Table 3. Primers used in the real-time PCR assay.

Gene		Oligonucleotide Sequence	Amplicon Length (bp)
For ATDC5			
Acan	Forward	5' GCTGCAGTG-TCTCAGAAGAAG 3'	104
	Reverse	5' GATGGTGAGGGAAGACCCTA 3'	
Alp	Forward	5' GACCCTGCCTTACCAACTCT 3'	123
	Reverse	5' TGGCTACATTGGTGTTGAGC 3'	
Col2a1	Forward	5' CCAGGATGCCCGAAAATTAG 3'	145
	Reverse	5' TTCTCCCTTGTCACCACGAT 3'	
Col10a1	Forward	5' CCTGGTTCATGGGATGTTTT 3'	181
	Reverse	5' CAGGAATGCCTTGTTCTCCT 3'	
Hprt	Forward	5' TGCTGACCTGCTGGATTACA 3'	118
	Reverse	5' TATGTCCCCCGTTGACTGAT 3'	
Ki67	Forward	5' CAAAAGGCGAAGTGGAGCTT 3'	105
	Reverse	5' TGTTTCGCAACTTTCGTTTGTG 3'	
Ocn	Forward	5' ACAAAGCCTTCATGTCCAAG 3'	215
	Reverse	5' TTTAGGGCAGCACAGGTC 3'	
For HPDC			
ACAN	Forward	5' CAACTACCCGGCCATCC 3'	158
	Reverse	5' GATGGCTGTAATGGAACAC 3'	
ALP	Forward	5' AGGGACATTGACGTGATCAT 3'	242
	Reverse	5' CCTGGCTCGAAGAGACC 3'	
COL2a1	Forward	5' GGATGGGCAGAGGTATAAATG 3'	368
	Reverse	5' GGGTCCCAGGTTCTCCATCT 3'	
HPRT	Forward	5' GCTGACCTGCTGGATTACAT 3'	260
	Reverse	5' CTTGCGACCTTGACCATCT 3'	
KI67	Forward	5' GGTGGGCACCTAAGACCTGAA 3'	235
	Reverse	5' TCCTAGGACTAGGAGCTGGAG 3'	
OCN	Forward	5' AGCCACCGAGACACCATGAGA 3'	288
	Reverse	5' CTCCTGAAAGCCGATGTGGTC 3'	
SOX9	Forward	5' CCACACTCCTCCTCCGGCATGA 3'	188
	Reverse	5' TCCACGTCGCGGAAGTCGAT 3'	

RESULTS

Active RA-sera inhibit cartilage matrix accumulation and matrix mineralization in ATDC5 micromasses

First we investigated the effect of RA-serum on osteochondrogenic differentiation of ATDC5 micromass cultures. Active RA-sera inhibited gene expression of *Ki67*, a marker of cell proliferation, by 40% at day 14 compared to remission RA-sera (Fig. 1a). Active RA-sera did not affect chondrogenic gene expression, i.e. *Col2a1* (Fig. 1b), *Acan* (Fig. 1c), and *Col10a* (Fig. 1d) in ATDC5 micromasses at day 14. Active RA-sera inhibited cartilage matrix accumulation in ATDC5 micromasses by 14% at day 14 compared to remission RA-sera (Fig. 1e,f).

Active RA-sera did not affect expression of the early osteogenic differentiation marker *Runx2* in ATDC5 micromass cultures at day 21 (Fig. 2a). Interestingly active RA-sera did inhibit *Alp* gene expression by 16% in ATDC5 micromasses (Fig 2b). It also did not affect the late osteogenic differentiation marker *Ocn* in these micromasses (Fig. 2c). On the other hand active RA-sera did inhibit matrix mineralization by 17% in ATDC5 micromasses at day 21 compared to remission RA-sera (Fig 2d,e).

Active RA-sera inhibit metabolic activity and matrix accumulation in HPDC micromasses

Since we found that active RA-sera inhibited cartilage matrix accumulation and matrix mineralization in ATDC5 micromasses, we also investigated the effect of active RA-sera compared to remission RA-sera in HPDCs. Active RA-sera did not affect *KI67* gene expression at day 7 (Fig. 3a), but it did inhibit metabolic activity of HPDCs by 8% at day 7 (Fig. 3b). Active RA-sera did not affect gene expression of *COL2a*1 (Fig. 3c) and *ACAN* (Fig. 3d) by HPDCs at day 7. However, active RA-sera did inhibit *SOX9* gene expression by 40% in HPDC micromasses at day 7 (Fig. 3e). Active RA-sera also inhibited cartilage matrix accumulation by 7% in HPDC micromass cultures at day 14 (Fig. 3f,g).

Active RA-sera did not affect gene expression of osteogenic differentiation markers *RUNX2* (Fig. 4a), *ALP* (Fig. 4b), and *OCN* (Fig. 4c) by HPDCs in micromass culture at day 21. Active RA-sera also did not affect matrix mineralization in HPDC micromasses at day 21 (data not shown). Both remission RA-sera and the healthy control serum pool similarly affected osteochondrogenic differentiation of HPDCs and matrix mineralization (data not shown).

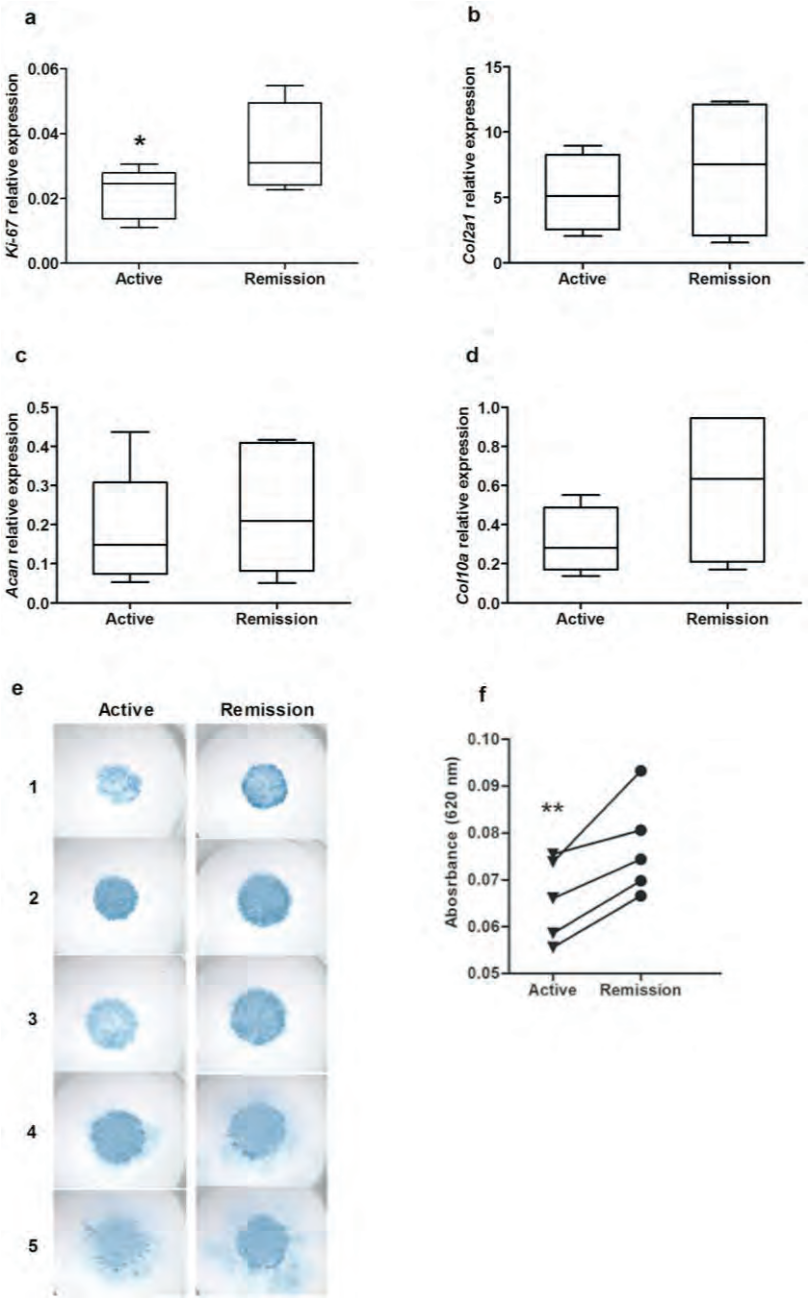

Figure 1. Active RA-sera inhibited proliferation and cartilage matrix accumulation in ATDC5 micromasses at day 14. (a) Active RA-sera inhibited *Ki67* gene expression. (b) Active RA-sera did not affect *Col2a1* gene expression. (c) Active RA-sera did not affect *Acan* gene expression. (d) Active RA-sera did not affect *Col10a* gene expression. (e) Active RA-sera

inhibited cartilage matrix accumulation (matrix stains blue by alcian blue staining). (f) Inhibitory effect of active RA-sera on cartilage matrix accumulation was also observed during quantitative analysis of cartilage matrix. Values are mean±SEM from experiments with sera obtained from 5 patients. Significant effect of active RA-sera, *p<0.05, **p<0.01.

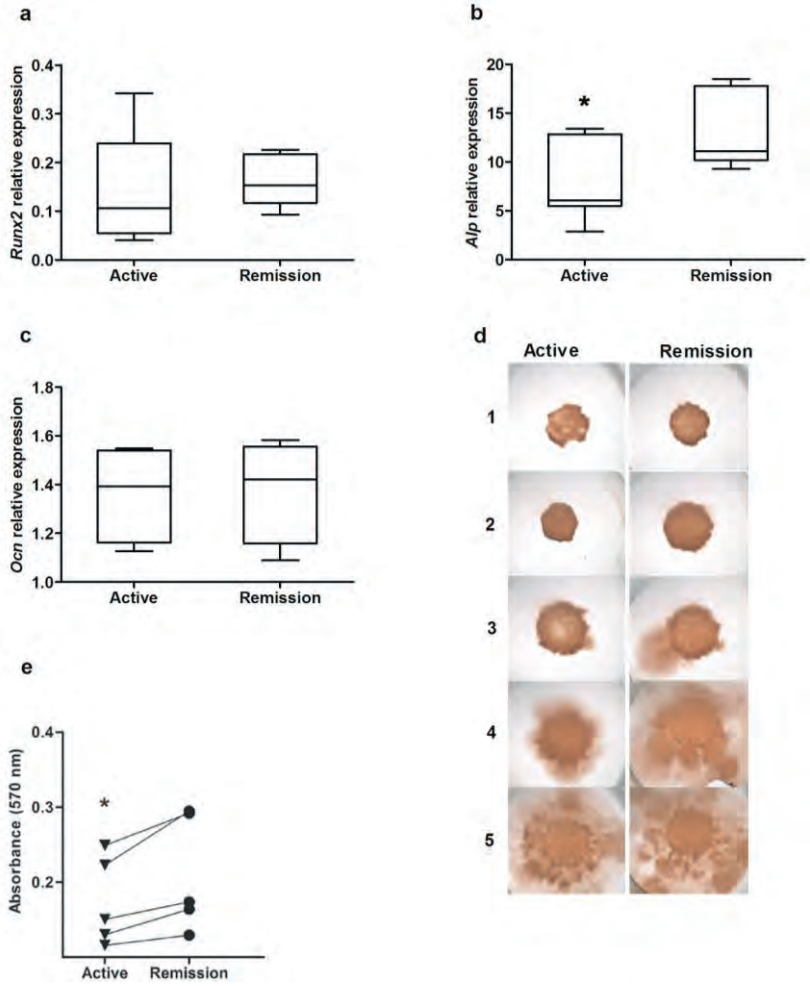

Figure 2. Active RA-sera inhibited matrix mineralization in ATDC5 micromasses at day 21. (a) Active RA-sera did not affect *Runx2* gene expression. (b) Active RA-sera inhibited *Alp* gene expression. (c) Active RA-sera did not affect *Ocn* gene expression. (d) Active RA-sera inhibited matrix mineralization (mineralized matrix stains red with alizarin red staining). (e) Inhibitory effect of active RA-sera on matrix mineralization was also observed during quantitative analysis of mineralized matrix. Values are mean±SEM from experiments with sera obtained from 5 patients. Significant effect of active RA-sera, *p<0.05.

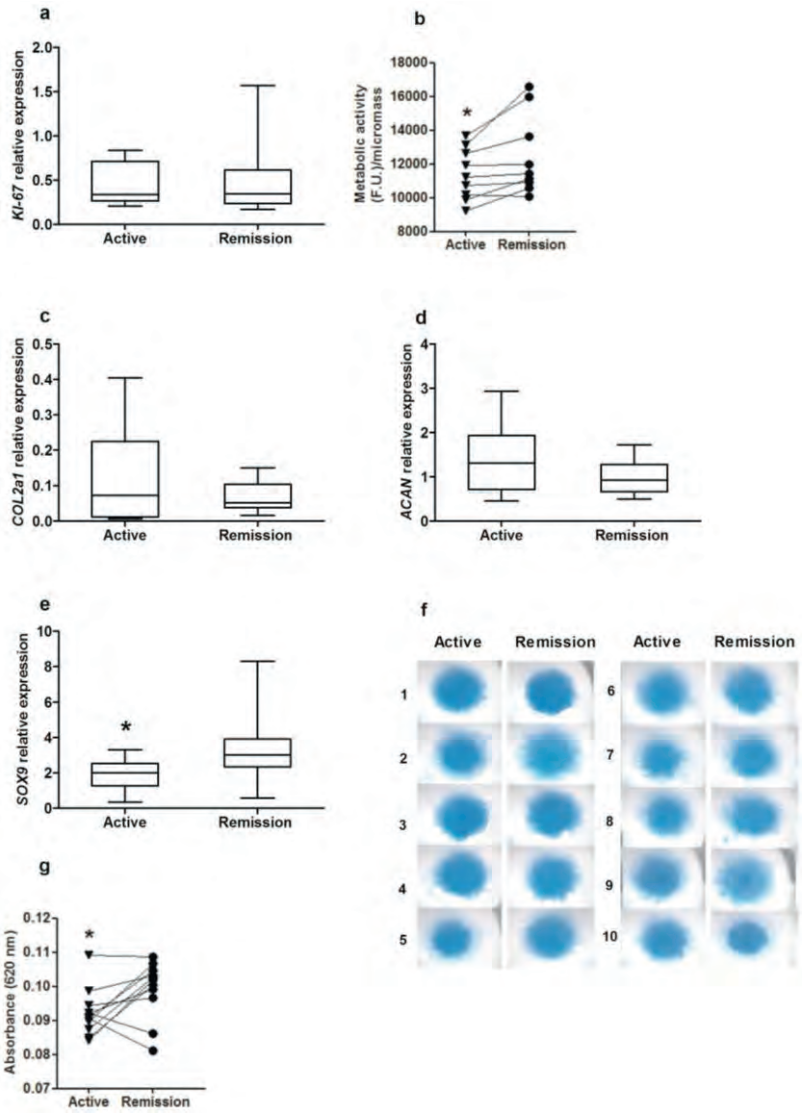

Figure 3. Active RA-sera inhibited not only metabolic activity but also cartilage matrix accumulation in HPDC micromasses. (a) Active RA-sera did not affect *KI67* gene expression at day 7. (b) Active RA-sera inhibited metabolic activity of HPDC at day 7 as analyzed by presto blue assay. (c) Active RA-sera did not affect *COL2a1* gene expression at day 7. (d) Active RA-sera did not affect *ACAN* gene expression at day 7. (e) Active RA-sera inhibited *SOX9* gene expression at day 7. (f) Active RA-sera inhibited cartilage matrix accumulation at day 14. (g) Inhibitory effect of active RA-sera on cartilage matrix accumulation was also observed during quantitative analysis of cartilage matrix. Values are mea±SEM from

experiments with sera obtained from 10 patients. Significant effect of active RA sera, *p<0.05.

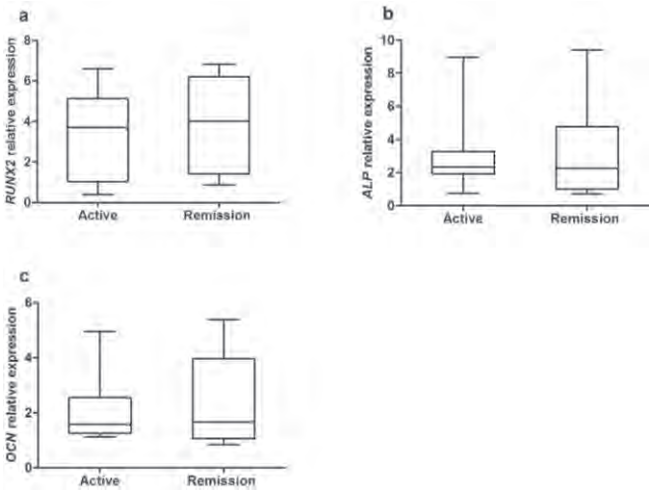

Figure 4. Active RA-sera did not affect on osteogenic differentiation in HPDC micromasses at day 21. (a) Active RA-sera did not affect *RUNX2* gene expression. (b) Active RA-sera did not affect *ALP* gene expression. (c) Active RA-sera did not affect *OCN* gene expression. Values are mean±SEM from experiments with sera obtained from 10 patients.

DISCUSSION

This study investigated the effect of active RA-sera on osteochondrogenic differentiation of two different precursor cell types, i.e. ATDC5 cells and HPDC, mimicking systemic inflammation as occurs during the active disease stage of RA. We found that sera from patients with active RA inhibit differentiation of osteochondrogenic precursor cells, as shown by decreased cartilage matrix accumulation and changes in osteochondral gene expression. This may explain, at least in part, delayed fracture healing in patients with chronic inflammation.

Active RA-sera decreased *Ki67* gene expression in ATDC5 cells, and metabolic activity in HPDCs. RA-serum contains elevated levels of IL-1β and TNFα as well as other inflammatory cytokines which are known to reduce the viability and proliferation of human chondrocytes *in vitro* (26, 33). Therefore our data suggests that sera from patients with active RA contain cytokines such as IL-1β and TNFα that inhibit proliferation of chondrogenic precursors which might explain the reduced callus formation and delayed fracture healing. Sera of patients with RA contain many factors that are differentially expressed compared to healthy control sera, e.g. Dickkopf-related protein 1, sclerostin, IL-1β, TNFα, and IL-6 (23, 34). Although we cannot distinguish with our current experimental set up which of these factors actually contributed to the inhibition of osteochondrogenic differentiation of progenitors, we can speculate that a cocktail of cytokines did play a role. Proinflammatory cytokines not only inhibit differentiation of chondrogenic precursors, but also restrict recovery of growth plate chondrogenesis and longitudinal bone growth (25, 27). Chronic inflammation inhibits cartilage matrix deposition in a murine fracture model (5, 6). Inhibition of systemic inflammation significantly accelerates fracture healing in rats (35). Moreover, inflammatory cytokines stimulate chondrogenic differentiation of precursors cells during the initial stage of fracture healing, but long term continuous treatment with inflammatory cytokines inhibits chondrogenic and osteogenic differentiation of precursor cells (25, 29). These studies indicate that individual inflammatory cytokines inhibit chondrogenic and osteochondrogenic differentiation (25-27). Recently we reported that active RA-sera inhibits osteoblast proliferation and differentiation, and enhances osteoblast-mediated osteoclastogenesis (14). In this study, we found that the combination of inflammatory factors present in RA-sera inhibits osteochondrogenic differentiation of precursors which might inhibit endochondral ossification and delay fracture healing.

Osteochondrogenic precursor cell line ATDC5 used in our study represents a well-established *in vitro* model to study endochondral bone formation (30, 36). The HPDC used in our study also has been phenotypically characterized (37). Osteochondrogenic differentiation of HPDC in micromass culture in the presence of 10% allogenic human serum, and the bone forming capacity of HPDCs *in vivo* has

already been shown (32). We found that RA-sera did inhibit matrix mineralization in ATDC5 but not in HPDC micromasses. This difference might be explained by differences in the cell populations we used, i.e. the ATDC5 murine cell line represents a homogeneous cell population, while the periost-derived cells represent a more heterogeneous population, containing fibroblasts, skeletal progenitors, and endothelial progenitors (38, 39). This difference might also be the result of differences in the degree of inflammation of the patients from whom the active RA-sera were obtained. The patients showed a higher degree of inflammation in the study using ATDC5 cells than in the study using HPDC cells cells based on a higher DAS 28 score, higher serum level of C-reactive protein, and a higher erythrocyte sedimentation rate (Table 2). Moreover, due to the limited volume of RA-sera, we were unable to analyze gene expression on multiple time points.

The strength of the study was the use of a well-defined patient group for RA-serum. We used well-established cell types, ATDC5 cells and HPDC, in a robust micromass model culture system of endochondral ossification. Periosteum-derived progenitor cells are superior to bone marrow-derived mesenchymal stem cells in their osteochondrogenic differentiation and callus formation potential during fracture healing (18). Also, in our experiments we tested active RA-serum, and used remission RA-serum from the same patient as control to reduce donor-dependent variation. During clinical remission the patients were taking DMARDs which could have reduced the inhibitory effect of active RA-serum on osteochondrogenic differentiation of precursors in our experiments, since DMARDs such as methotrexate have been shown to affect bone formation and delay fracture healing (40, 41). On the other hand, Satoh et al. have shown that a therapeutical dose of methotrexate does not influence fracture healing, making this an unlikely source of bias (42). Elevated cytokine levels in RA-serum have already been described (23, 24). We purposely first aimed to identify specific patients, or groups of patients, who's complete serum, rather than the individual cytokines, has a clear differential effect on osteochondrogenic differentiation of precursor cells. It would be interesting to analyze the cytokine profiles in this RA-serum to further interpret the effects observed in our study, but such an analysis requires much more serum than currently available. It would not do to simply quantify and report the concentrations of a limited set of cytokines, since the effect of the individual cytokines may vary considerably from the effect of a complex combination of cytokines, growth factors, and antagonists present in human serum (43).

A limitation of our study is possible differences in the inhibitory potential of the serum on osteochondrogenic differentiation as a result of differences in storage time. The potency of serum decreases with storage time, and therefore active RA-sera stored for more than one year might have lost more of its potency than sera stored for a shorter time, resulting in, if anything, an underestimation of the effect of

active RA-sera on osteochondrogenic differentiation of ATDC5 cells and HPDC. Another limitation might be the relatively low number of RA patients included. Statistical significance between groups is not easily obtained due to a lower number of patients included.

Conclusion

The current study shows that active RA-sera inhibit osteochondrogenic differentiation of precursor cells. These findings provide important new insight regarding the role of factors present in the serum of patients with systemic inflammation in delayed fracture healing, and suggest that mitigation of systemic inflammation, by agents that do not affect fracture healing directly themselves (e.g. COX2 inhibitors), might rescue delayed fracture healing in patients with systemic inflammatory disease.

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CHAPTER 4

Inflammatory Factors in the Circulation of Patients with Active Rheumatoid Arthritis Stimulate Osteoclastogenesis via Endogenous Cytokine Production by Osteoblasts

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ABSTRACT

Purpose. Generalized bone loss, as occurs in patients with rheumatoid arthritis (RA), is related to elevated levels of circulating cytokines. Individual cytokines have deleterious effects on proliferation and differentiation of osteoblast cell lines, but little is known about the effect of the interaction between inflammatory factors in the circulation of patients with active RA on human osteoblast function, including their communication towards other bone cells. We investigated whether serum from patients with active RA enhances cytokine production by osteoblasts, thereby effectively altering osteoblast-stimulated osteoclastogenesis.
Methods. Serum was obtained from 20 patients with active RA (active RA-sera) and from the same patients in clinical remission (remission RA-sera). To determine osteoclastogenesis, RA-sera–pretreated primary human osteoblast cultures were established in direct contact with human osteoclast precursors in the presence or absence of OPG or IL-6 inhibitor.
Results. Compared to remission RA-sera, active RA-sera inhibited osteoblast proliferation and differentiation *in vitro* as demonstrated by a reduced DNA content and gene expression of KI-67, collagen type 1, osteopontin, and osteocalcin. Active RA-sera inhibited osteoprotegerin expression and enhanced RANKL and IL-6 expression, but did not alter IL-8 expression in osteoblasts. IL-1β, IL-17 and TNFα expression were undetectable. In co-culture, active RA-sera treatment of osteoblasts stimulated, while addition of OPG or IL-6 inhibitory antibodies significantly reduced the number of osteoclasts.
Conclusion. Active RA-sera contain circulating factors, likely cytokines and chemokines that might contribute to bone loss by directly inhibiting osteoblast proliferation and differentiation, but especially these factors modulate endogenous cytokine production by osteoblasts, thereby affecting osteoclastogenesis.

KEYWORDS:
Osteoporosis, bone, IL-6, RANKL, OPG, osteogenic differentiation

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease of unknown etiology, characterized by local bone erosion, joint space narrowing, and extra-articular manifestations such as generalized osteoporosis (1, 2). Early stage RA patients show decreased bone mineral density (BMD) as well as a 2-fold increased risk of vertebral and non-vertebral (including hip) fractures compared to healthy subjects (3-5). Such fractures are associated with severe morbidity and increased mortality rate (6). Clinical studies have suggested that the diagnosis of RA at an early stage of the disease, and treatment by disease modifying anti-rheumatic drugs (DMARDs) prevents bone loss and "repairs" bone (7, 8). However the molecular mechanisms responsible for generalized bone loss in RA are still not fully understood.

Generalized osteoporosis in RA is in part caused by immobility and corticosteroid therapy (9), but it has also been attributed to the effects of chronic inflammation, such as elevated levels of circulating cytokines. Cells in inflamed synovia in RA produce high amounts of growth factors, as well as cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-17 (IL-17), tumor necrosis factor-α (TNFα), and CXCL8 (IL-8) (10). Elevated levels of these cytokines are found in synovial fluid and serum from RA patients (11, 12). Each of these cytokines has been shown to directly affect osteoblast as well as osteoclast formation and activity, thus disturbing the delicate balance between bone formation and resorption during bone remodeling (13-17). In addition, TNFα and IL-1β affect the production of osteoblast and osteoclast modulatory-factors by osteocytes, which are terminally differentiated cells of the osteoblast lineage, and IL-1β strongly stimulates osteocyte-stimulated osteoclastogenesis (14, 18). Thus cytokines are potentially able to affect bone mass by altering the production of signaling factors by cells of the osteoblast lineage. Interestingly these signaling factors may comprise of endogenous cytokines, since osteoblasts and osteocytes have been shown to produce cytokines such as IL-1β and IL-6 in sufficient quantities to alter osteoclastogenesis *in vitro* (19, 20). In other words, cytokines are locally produced around inflamed joints in RA and end up in the circulation of patients, which may trigger ubiquitous cytokine production within bone. Unfortunately it is currently unknown whether the complex combination of inflammatory factors that is actually present in the circulation of RA patients is equally potent as individual recombinant cytokines in modulating osteoblast signaling. The effect of a combination of these proteins might well be divergent from the observed effect of individual cytokines on bone metabolism.

We hypothesized that circulating inflammatory mediators in patients with active RA contribute to bone loss by affecting not only osteoblast proliferation and differentiation, but also osteoblast-to-osteoclast communication, through the

production of endogenous cytokines. In our study we did not test individual cytokines, but we cultured human primary osteoblasts with serum obtained from 20 well characterized patients with active early RA before starting disease-modifying anti-rheumatic drugs (DMARDs) therapy, as well as with serum obtained from these same 20 patients during clinical remission. We analyzed the effect of sera on osteoblast proliferation and differentiation, and on the ability of osteoblasts to support osteoclast formation from human peripheral blood mononuclear cells cultured in direct cell-cell contact with the serum-pretreated osteoblasts.

METHODS

Selection of RA patients and serum collection

Twenty RA patients were recruited from the CareRA project coordinated by the clinical rheumatology team of the Skeletal Biology and Engineering Research Center, KU Leuven, Belgium (mean age: 51.4±13.3 yrs; 16 females, 4 males). In CareRA patients with RA according to the 1987 classification criteria were recruited at an early stage of the disease (less than 1 year disease duration) and before they had taken DMARDs or corticosteroids. Blood samples were collected, and within 1 h centrifuged for 10 min at 3000 rpm to separate the sera, that were aliquoted and stored at –80ºC. Patient characteristics, demographics, and clinical data (anti-citrullinated protein antibodies, rheumatoid factor, erosions) were collected at baseline (Table 1). Then standardized treatment was started with methotrexate (MTX) or a combination of MTX with sulphasalazine or leflunomide and corticosteroids for 34 weeks. After this standardized treatment for 34 weeks, corticosteroid treatment was stopped, and in those patients that had also received DMARD combination therapy at baseline, DMARD treatment was stepped down to a monotherapy with MTX from week 40 onwards. At 52 weeks, blood samples were collected once again, and sera were separated from the blood samples, and stored as described above. Patients with thyroid dysfunction, other inflammatory diseases than RA, and/or pregnancy were excluded from this study. Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and DAS28CRP were measured at baseline when patients had active untreated disease, and after 52 weeks when the disease activity was controlled with medication (Table 2). Individual healthy control sera were obtained from two healthy donors. In addition, a healthy control sera pool was made by pooling the sera from 22 healthy donors (age: 40.5±17.7 yrs; 14 females, 8 males). The serum CRP level of the individual healthy control sera, as well as the healthy control pool was <2.5 mg/l. This study was approved by the local ethical committee of the Leuven University Hospital, Belgium, and the Ethical Review Board of the VU University Medical Center, Amsterdam, The Netherlands. All subjects gave informed consent.

Human primary osteoblast culture

Trabecular bone samples (surgical waste) from 2 male donors (age: 42 and 61 yrs) were obtained from the anterior iliac crest during sinus floor elevation surgery using autologous anterior iliac crest bone graft. The serum CRP level of the donors was <2.5 mg/l. The protocol was approved by the Ethical Review Board of the VU University Medical Center, Amsterdam, The Netherlands, and all subjects gave informed consent.

Table 1. Characteristics and demographics of the 20 patients included in this study

	active RA
Sex (female/male)	16/4
Age, yrs	51.4 ± 13.3
Weight, kg	72.1 ± 10.3
BMI, kg/m^2	25.8 ± 3.8
# anti-CCP positive patients	15
# RF-positive patients	13
# patients with erosions	6
# patients with NSAIDs medication	18

Values are mean ± SD. Twenty patients were included in this study.
BMI, body mass index; anti-CCP, anti-citrullinated protein antibodies; RF, rheumatoid factor; NSAIDs, non-steroidal anti-inflammatory drugs.

Table 2. Clinical data of the patients included in this study at baseline and at 52 weeks

	baseline (n=20)	52 weeks (n=20)	p value
DAS28 score	5.1 ± 1.2	2.0 ± 0.6	<0.0001*****
CRP (mg/l)	28.6 ± 48.4	4.8 ± 6.7	0.0176*****
ESR (mm/h)	47 ± 31	20.8 ± 11.4	0.0005*****

Values are mean ± SD. n, number of patients. *Significant effect of disease modifying anti-rheumatic drugs, $p < 0.05$. DAS28, disease activity score; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

Human primary osteoblast cultures were established as described earlier (21). Briefly, trabecular bone fragments were placed in sterile phosphate-buffered saline (PBS), chopped into small fragments, and washed extensively with PBS. Bone fragments were then incubated with 2 mg/ml collagenase type II (Worthington, Freehold, NJ) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY)/Nutrient mixture F12 (F12) (DMEM/F12, 1:1 (vol/vol)) for 2 h at 37°C in a shaking water bath to remove all adhering cells from the bone chip surfaces. Bone fragments were then washed with medium containing 10% Fetal Clone I serum (HyClone), subdivided into equal portions, and transferred to 25 cm^2 or 75 cm^2 culture flasks (Nunc, Roskilde, Denmark). To obtain outgrowth of bone cells, bone fragments were cultured in DMEM/F-12 supplemented with 10% Fetal Clone I serum, 100 U/ml penicillin (Sigma, Hamburg, Germany), 100 µg/ml streptomycin sulfate (Gibco), 50 µg/ml gentamicin (Gibco), 1.25 µg/ml fungizone (Gibco), and 100 µg/ml ascorbate (Merck, Darmstadt Germany) at 37°C in a

humidified atmosphere with 5% CO_2. The culture medium was refreshed twice a week. After reaching subconfluency, outgrowth bone cells were trypsinized with 0.25% trypsin (Difco Laboratories, Detroit, MI) and 0.1% EDTA (Sigma) in PBS. Cells were seeded at a density of $1x10^4$ cells/well of a 24-well culture plate (Greiner Bio-One, Frickenhausen, Germany) and incubated overnight. Then the medium was replaced by DMEM/F-12 with 0.1% healthy control sera pool, and the cells were incubated overnight again. The overnight incubation with 0.1% serum (serum-deprived medium) resulted in synchronization of the primary human osteoblasts (22).

Osteoblast culture with RA-sera

After synchronization of primary human osteoblasts, cell culture was continued for 3, 7, or 10 days in DMEM/F12 medium containing either 10% active-RA-sera, remission-RA-sera, or healthy control sera. Culture medium with 10% RA-sera or healthy control sera was filtered using 0.22 µm filter (Millipore, Amsterdam, The Netherlands) before adding to the cell cultures. RNA was isolated, and cell lysates were collected at day 3, 7 and 10.

Total DNA content

Osteoblasts were washed with PBS, lysed in 250 µl ice-cold water, sonicated twice for 10 sec, and centrifuged at 5000 rpm for 5 min at room temperature. The total DNA content of the cell layer was quantified using a Cyquant Cell Proliferation Assay (Molecular Probes, Eugene, OR) according to the manufacturer's protocol.

Alkaline phosphatase (ALP) activity and total protein analysis

To determine ALP acitvity, osteoblasts were washed with PBS, lysed in 250 µl ice-cold water, sonicated twice for 30 sec at 50/60 Hz, centrifuged at 5000 rpm for 5 min at room temperature, and the supernatant was collected. ALP activity was measured in the supernatant and analyzed using an ALP IFCC liquid assay (Roche, Basel, Switzerland), based on the method as described by Lowry (23), according to manufacturer's instructions. ALP activity was expressed in micromole per µg protein. Quantification of the total amount of protein was performed using a BioRad protein assay (BioRad, Hercules, CA) according to the manufacturer's protocol.

RNA isolation and real-time RT-PCR

Total RNA was isolated using an RNeasy® Micro kit with an on-column DNase I digestion (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Total RNA concentrations were measured with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA synthesis was performed in a thermocycler GeneAmp® PCR System 9700 PE (Applied Biosystems, Foster City,

CA), using a SuperScript® VILO™ cDNA Synthesis Kit (LifeTechnologies, Bleiswijk, The Netherlands), with 0.1 µg of total RNA in 20 µl reaction mixture consisting of VILO Reaction Mix and SuperScript Enzyme Mix. cDNA was stored at −20°C until real-time PCR analysis.

Real-time PCR reactions were performed using 2.5 µl cDNA and SYBR® Green Supermix (Roche Laboratories, Indianapolis, IN) according to the manufacturer's instructions in a LightCycler® (Roche Diagnostics, Switzerland). For quantitative real-time PCR, the values of relative target gene expression were normalized to the relative mean of YWHAZ and HPRT housekeeping gene expression. Real-time PCR was used to assess expression of the following genes: KI-67, RUNX2, ALP, collagen 1 (COL1), osteopontin (OPN), osteonectin (ON), osteocalcin (OCN), DMP1, IL-1β, IL-6, IL-8, IL-17, macrophage colony stimulating factor (MCSF), osteoprotegerin (OPG), Receptor activator of nuclear factor-kappa B ligand (RANKL), Sclerostin (SOST), DKK1 and TNFα. All primers used for real-time PCR were from Life Technologies, Bleiswijk, The Netherlands. The primer sequences are listed at Table 3. In each assay for osteogenic marker gene expression, mRNA preparations of human bone were used as a reference and internal control for the primer sets to pick up the specific mRNA of interest.

Co-culture of RA-sera–pretreated osteoblasts and osteoclast precursors; osteoclastogenesis assay

Subconfluent human primary osteoblasts were trypsinized with 0.25% trypsin (Difco Laboratories, Detroit, MI) and 0.1% EDTA (Sigma) in PBS. – Cells were seeded at a density of $7x10^3$ cells/well of a 48-well culture plate (Greiner Bio-One, Frickenhausen, Germany), and incubated overnight in DMEM/F12 with 10% fetal calf serum (FCS). Then the medium was replaced by DMEM/F12 with either 10% active RA-sera, remission RA-sera, or healthy control sera for 10 days. To stimulate osteogenic differentiation of the primary human osteoblasts, 10 nM of 1α,25-dihydroxyvitamin D_3 (Sigma Aldrich) was added during the first 3 days of culture.

Peripheral blood mononuclear cells (PBMCs) were isolated from a buffy coat (Sanquin, Amsterdam, The Netherlands) as described previously (24). Briefly, buffy coats were diluted 1:1 in Hank's buffered salt solution (HBSS; Gibco, Paisley, UK). Twenty-five ml of diluted blood was layered on 15 ml lymphoprep (Axisshield Po CAS, Oslo, Norway), and centrifuged for 30 min at 1200xg without brake to create different cell layers. The interphase containing PBMCs was collected and resuspended in HBSS. PBMCs were washed twice in HBSS, and recovered in DMEM containing 10% FCS and antibiotics (100 U/ml penicillin (Sigma, St. Louis, MO), 100 g/ml streptomycin (Sigma), and 250 ng/ml amphotericin B (Sigma)).

Medium containing RA-sera was removed from the osteoblast cultures. After washing the osteoblasts with PBS, PBMCs in DMEM containing 10% FCS

were seeded at $5x10^5$ cells per well directly on top of the RA-sera–pretreated osteoblasts in 48-well culture plates. RANKL was blocked in osteoblasts pretreated with sera from 11 donors by adding 0.5 µg/ml recombinant human OPG (Peprotech, London, UK). IL-6 was blocked in osteoblasts pretreated with sera from 7 donors by adding 0.5 µg/ml of human IL-6 antibody (Clone #6708, R&D Systems). IgG isotype control (Clone # 11711, R&D Systems) was added as a control for the IL-6 antibody. After 3 days of culture, the medium was replaced with DMEM containing 10% FCS, 100 U/ml penicillin, 100 g/ml streptomycin, and 250 ng/ml amphotericin B. After 3 weeks of culture, the cells were fixed in 4% formaldehyde, and stained for tartrate-resistant acid phosphatase (TRAcP) according to manufacturer's instructions (Sigma). Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) staining. Osteoclast formation was assessed by counting the number of TRACP-positive multinucleated cells (MNCs), containing 3 or more nuclei per cell. Osteoclasts were counted on 10 fixed microscopic fields of each well using a Leica DM IL microscope (Leica, Wetzlar, Germany) equipped with a 20x objective.

Statistical analysis

Data are expressed as mean. Data of gene expression and total DNA by active and remission RA-sera treated osteoblasts were normalized for mean data from control sera treated osteoblasts. The effects of active RA-sera and remission RA-sera on osteoblasts and on osteoblast-mediated osteoclastogenesis, as well as the effects of OPG and IL-6 inhibitor on active RA-sera or remission RA-sera–pretreated osteoblast-mediated osteoclastogenesis were tested using a two-tailed paired t-test. The effects of active RA-sera or remission RA-sera on osteoblast-mediated osteoclastogenesis compared with the effects of healthy control sera was tested by one-way variance of analysis (ANOVA) followed by Bonferroni's multiple comparison test. Differences were considered significant if $p<0.05$. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA).

Table 3. Primers used in the real-time PCR assay

Gene		Oligonucleotide Sequence	Amplicon length (bp)
HPRT1	Forward	5' GCTGACCTGCTGGATTACAT 3'	260
	Reverse	5' CTTGCGACCTTGACCATCT 3'	
YWHAZ	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'	
KI67	Forward	5' GGTGGGCACCTAAGACCTGAA 3'	235
	Reverse	5' TCCTAGGACTAGGAGCTGGAG 3'	
RUNX2	Forward	5' ATGCTTCATTCGCCTCAC 3'	156
	Reverse	5' ACTGCTTGCAGCCTTAAAT 3'	
ALP	Forward	5' AGGGACATTGACGTGATCAT 3'	242
	Reverse	5' CCTGGCTCGAAGAGACC 3'	
COL1	Forward	5' TCCAACGAGATCGAGATCC 3'	191
	Reverse	5' AAGCCGAATTCCTGGTCT 3'	
Osterix	Forward	5' GGCAAGTTCACTCGTTC 3'	218
	Reverse	5' GTCTGACTGGCCTCCTCTTC 3'	
OPN	Forward	5' TTCCAAGTAAGTCCAACGAAAG 3'	181
	Reverse	5' GTGACCAGTTCATCAGATTCAT 3'	
ON	Forward	5' TACATCGGGCCTTGCAAATAC 3'	101
	Reverse	5' AGGGTGACCAGGACGTTCTTG 3'	
DMP1	Forward	5' TTGGCTAGCTGGTGGCTTCT 3'	375
	Reverse	5' AACTCGGAGCCGTCTCCAT 3'	
OCN	Forward	5' AGCCACCGAGACACCATGAGA 3'	288
	Reverse	5' CTCCTGAAAGCCGATGTGGTC 3'	
DKK1	Forward	5' GCATGCGTCACGCTATGT 3'	271
	Reverse	5' TTACAGATCTTGGACCAGAA 3'	
IL-1β	Forward	5' TGGAGCAACAAGTGGTGTTCT 3'	270
	Reverse	5' GAGAGGTGCTGATGTACCAGTT 3'	
IL-6	Forward	5' ACAGCCACTCACCTCTTCA 3'	207
	Reverse	5' ACCAGGCAAGTCTCCTCAT 3'	
IL-8	Forward	5' TCTGCAGCTCTGTGTGAAG 3'	147
	Reverse	5' TGTGTTGGCGCAGTGTGG 3'	
MEPE	Forward	5' CTACAACCGATCCACCTCAC 3'	255
	Reverse	5' ACAATCGGGGTGACACAGGT 3'	
M-CSF	Forward	5' CCGAGGAGGTGTCGGAGTAC 3'	100
	Reverse	5' AATTTGGCACGAGGTCTCCAT 3'	
OPG	Forward	5' TGGAATAGATGTTACCCTGTGTG 3'	298
	Reverse	5' GCTGCTCGAAGGTGAGGTTA 3'	
RANKL	Forward	5' CATCCCATCTGGTTCCCATAA 3'	60
	Reverse	5' GCCCAACCCCGATCATG 3'	
SOST	Forward	5' GGGTGGCAGGCGTTCA 3'	164
	Reverse	5' CTGTACTCGGACACGTCTTTGGT 3'	
TNFα	Forward	5' AGAGGGCCTGTACCTCATCT 3'	315
	Reverse	5' AGGGCAATGATCCCAAAGTAG 3'	

RESULTS

Active RA-sera inhibit osteoblast proliferation and differentiation

To investigate the effect of the combination of circulating cytokines on osteoblast proliferation, serum form active and remission RA patients was added to primary human osteoblast cultures. Active RA-sera did not affect KI-67 gene expression in osteoblasts at day 3, but reduced KI-67 gene expression by 45% at day 7, and by 29% at day 10 compared with remission RA-sera (Fig. 1a). Active RA-sera also reduced the total DNA content in osteoblast cultures by 14% at day 10 (Fig. 1b). Remission RA-sera did not affect osteoblast proliferation, differentiation, or cytokine gene expression in osteoblasts compared with healthy control sera (data not shown).

We then investigated the effect of RA-sera on osteoblast differentiation, which was assessed by quantifying the expression of genes specifically associated with the osteogenic lineage. Active RA-sera reduced gene expression of the early osteoblast differentiation marker COL1 by 17% at day 3, and by 12% at day 7 compared with remission RA-sera (Fig. 1c). Active RA-sera reduced OPN gene expression by 22% at day 7 (Fig. 1d). Active RA-sera reduced gene expression of the late osteoblast differentiation marker OCN by 31% at day 3, by 37% at day 7, and by 24% at day 10 (Fig. 1e). Active RA-sera reduced ON gene expression by 28% and DMP1 gene expression by 34% at day 10 (Fig. 1f). Active RA-sera did not affect ALP activity and gene expression of RUNX2 and ALP by osteoblasts (data not shown). The osterix gene did not anneal with the primer set used in our qPCR.

Active RA-sera affect cytokine and growth factor/inhibitor gene expression in osteoblasts

Active RA-sera affected cytokine gene expression by osteoblasts. It increased IL-6 gene expression by 32% in osteoblasts (Fig. 2a), but it did not affect IL-8 gene expression in these cells compared with remission RA-sera (Fig 2b). Active RA-sera increased RANKL gene expression by 90% at day 10 (Fig. 2c), while it reduced OPG gene expression by 18% at day 10 (Fig. 2d). As a result, active RA-sera increased the ratio of RANKL/OPG gene expression by 48% at day 3, and by 100% at day 10 (Fig. 2e). Human osteoblasts expressed DKK1 and SOST gene, but active RA-sera did not affect DKK1 and SOST gene expression at day 10 (Fig 2f). IL-1β, IL-17 and TNFα gene expression in osteoblasts was below the detection limit.

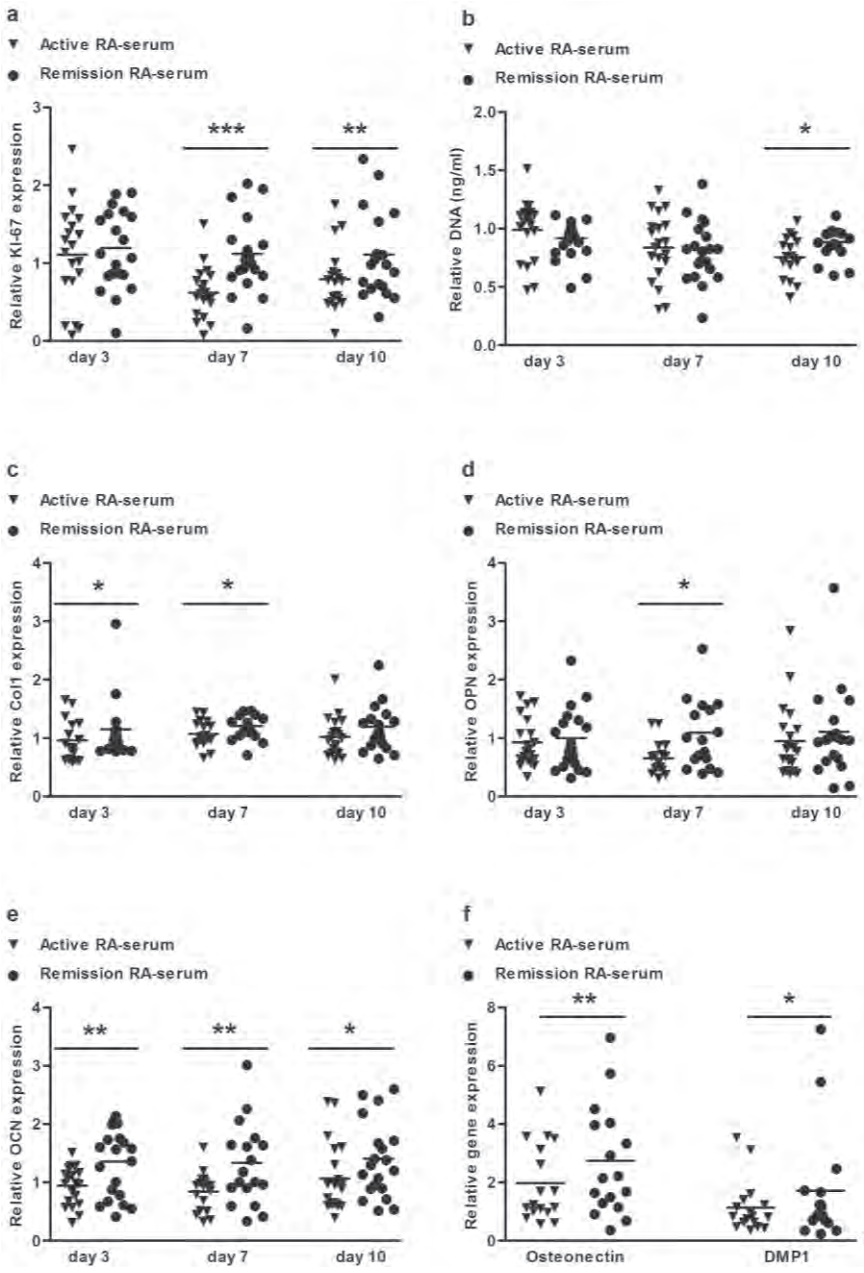

Figure 1. Active RA-sera inhibited osteoblast proliferation compared to remission RA-sera as well as gene expression of early and late osteogenic markers in osteoblasts. **a** Active RA-sera inhibited KI-67 gene expression at day 7 and 10. **b** Active RA-sera inhibited total DNA content at day 10. **c** Active RA-sera inhibited COL1 gene expression at day 3 and 7. **d**

Active RA-sera inhibited OPN gene expression at day 7. **e** Active RA-sera inhibited OCN gene expression at day 3, 7, and 10. **f** Active RA-sera inhibited ON and DMP1 gene expression at day 10. To minimize the osteoblast donor-dependent variation, values are plotted as relative to mean value from control sera-treated osteoblasts. Values are mean. Sera were obtained from 20 patients. Significant effect of active RA-sera, * p<0.05, ** p<0.01, *** p<0.001. COL1, collagen 1; OPN, osteopontin; OCN, osteocalcin.

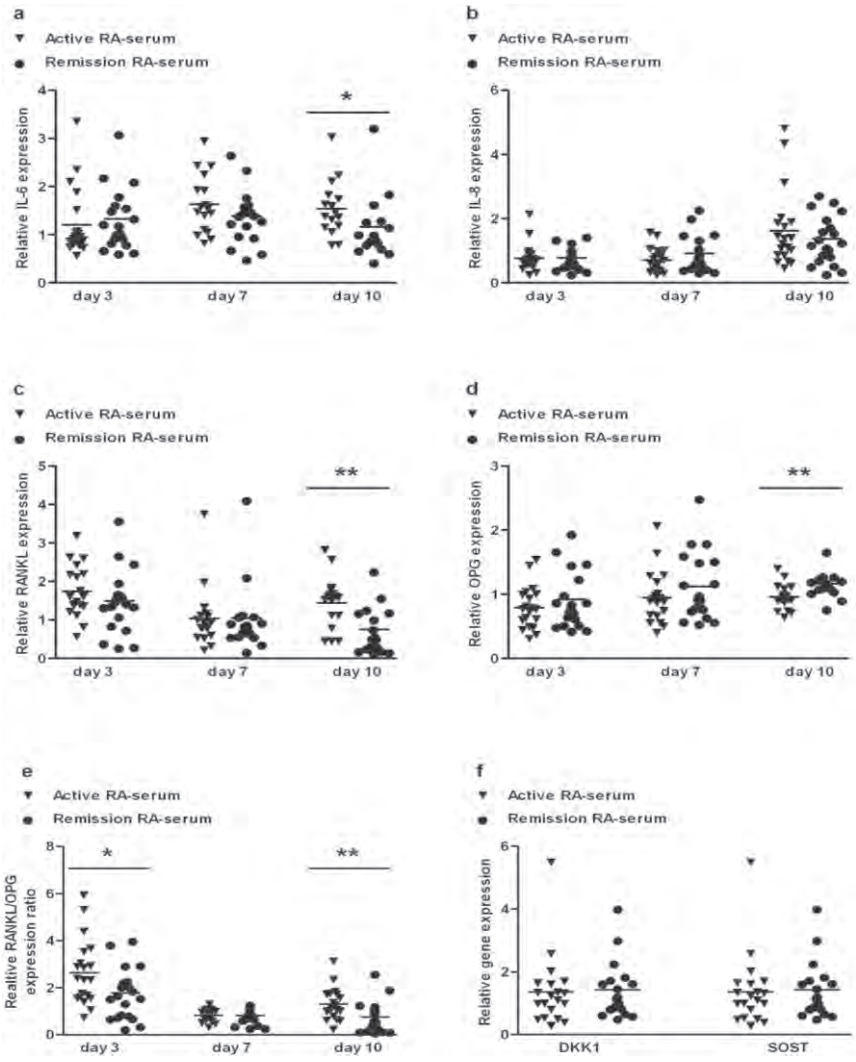

Figure 2. Active RA-sera affected RANKL, OPG and IL-6 gene expression in osteoblasts. **a** Active RA-sera enhanced IL-6 gene expression at day 10. **b** Active RA-sera did not affect IL-8 gene expression. **c** Active RA-sera enhanced RANKL gene expression at day 10. **d** Active RA-sera inhibited OPG gene expression at day 10. **e** Active RA-sera increased the gene

expression of RANKL/OPG ratio at day 3 and 10. **f** Active RA-sera did not affect DKK1 and SOST gene expression at day 10**.** To minimize the osteoblast donor-dependent variation, values are plotted as relative to mean value from control sera-treated osteoblasts. Values are mean from 2 independent experiments with sera obtained from 20 patients. Significant effect of active RA-sera, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. RANKL, receptor activator of nuclear factor-kappaB ligand; OPG, osteoprotegerin; IL-6, interleukin 6.

Active RA-sera enhance osteoblast-mediated osteoclastogenesis via RANKL and IL-6

Since active RA-sera enhanced the RANKL/OPG ratio and IL-6 gene expression in osteoblasts, which both enhance osteoclastogenesis, we then investigated the effect of endogenous cytokines produced on osteoblast-osteoclast communication by co-culturing osteoclast precursors with active RA-sera–pretreated osteoblasts. TRACP-positive multinucleated cells (MNC) were categorized in two groups, i.e. osteoclasts with 3-5 nuclei, and osteoclasts with >5 nuclei. PBMCs did not form osteoclasts in the absence of osteoblasts (Fig. 3a). PBMCs directly co-cultured with osteoblasts that were pretreated with healthy control sera formed less osteoclasts (Fig. 3b) compared with PBMCs co-cultured with remission RA-sera–pretreated osteoblasts (Fig. 3c), or active RA-sera–pretreated osteoblasts (Fig. 3d). Pretreatment of osteoblasts with active RA-sera increased the number of osteoclasts with 3-5 nuclei by 34% compared with pretreatment of osteoblasts with remission RA-sera, and by 170% compared with pretreatment of osteoblasts with healthy control sera (Fig. 3e). Active RA-sera–pretreated osteoblasts increased the number osteoclasts with >5 nuclei by 180% compared with healthy control sera-pretreated osteoblasts (Fig. 3e). Similarly, remission RA-sera–pretreated osteoblasts increased the number of osteoclasts with 3-5 nuclei by 100% compared with healthy control sera-pretreated osteoblasts (Fig. 3e). OPG treatment of active RA-sera–pretreated osteoblasts and PBMCs in co-culture reduced the number of osteoclasts with 3-5 nuclei by 36%, and the number of osteoclasts with >5 nuclei by 47% (Fig. 4a). IL-6 antibody also reduced the number of osteoclasts with 3-5 nuclei by 29%, and the number of osteoclasts with >5 nuclei by 44% in the presence of active RA-sera–pretreated osteoblasts (Fig. 4b). Addition of neither OPG nor IL-6 antibody reduced the number of osteoclasts that developed in the presence of remission RA-sera–pretreated osteoblasts (Fig. 4a,b), or in the presence of healthy control sera-pretreated osteoblasts (data not shown). Addition of IgG isotype control for IL-6 did not affect the number of osteoclasts that developed in healthy control sera-pretreated osteoblasts (data not shown).

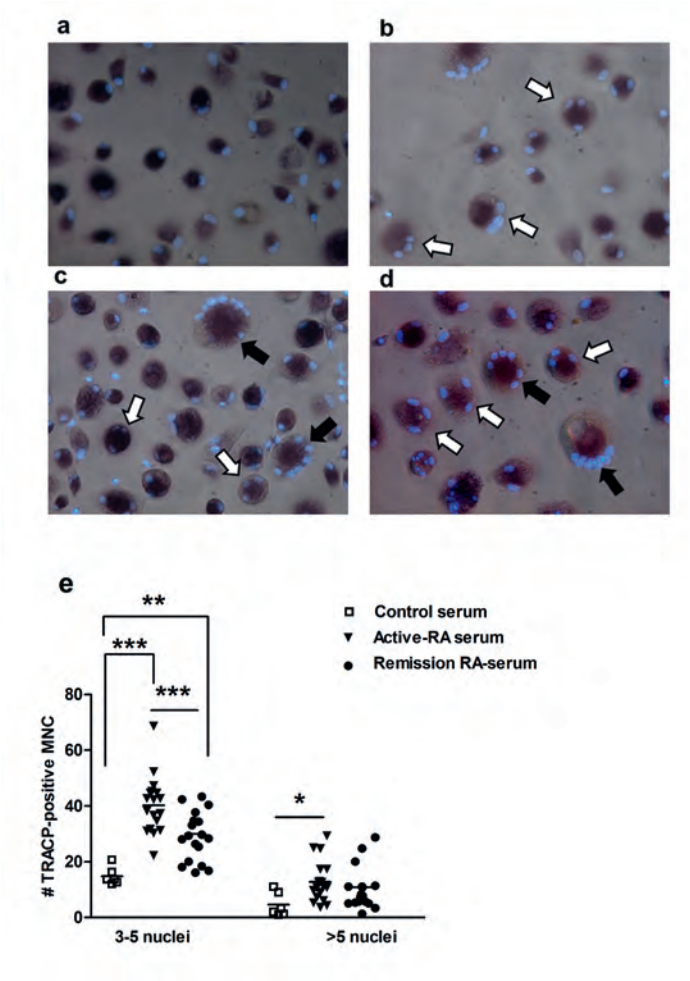

Figure 3. Active RA-sera enhanced osteoblast-mediated osteoclastogenesis compared with remission RA-sera and healthy control sera in co-cultures of osteoblasts and PBMCs. **a** PBMCs alone did not form TRACP-positive osteoclasts. **b** Healthy control sera-pretreated osteoblasts resulted in the formation of TRACP-positive osteoclasts in co-cultures of osteoblasts and PBMCs (white arrow; osteoclast with 3-5 nuclei). **c** Remission RA-sera–pretreated osteoblasts resulted in the formation of more TRACP-positive osteoclasts in co-cultures of osteoblasts and PBMCs compared with healthy control sera-pretreated osteoblasts (white arrow: osteoclast with 3-5 nuclei; black arrow: osteoclast with >5 nuclei). **d** Active RA-sera–pretreated osteoblasts resulted in the formation of more TRACP-positive osteoclasts compared with remission RA-sera and healthy control sera-pretreated osteoblasts (white arrow: osteoclast with 3-5 nuclei; black arrow: osteoclast with >5 nuclei). **e** Active RA-sera–pretreated osteoblasts enhanced osteoclastogenesis compared to remission RA-sera and healthy control sera-pretreated osteoblasts. Remission RA-

sera–pretreated osteoblasts also enhanced osteoclastogenesis compared with healthy control sera-pretreated osteoblasts. Values are mean from 2 independent experiments with sera obtained from 20 patients. Magnification: 20x. PBMCs, peripheral blood mononuclear cells; TRACP-positive MNC, TRACP-positive multinucleated cells. Significant effect of active and remission RA-sera, * p<0.05, ** p<0.01, *** p<0.001**.**

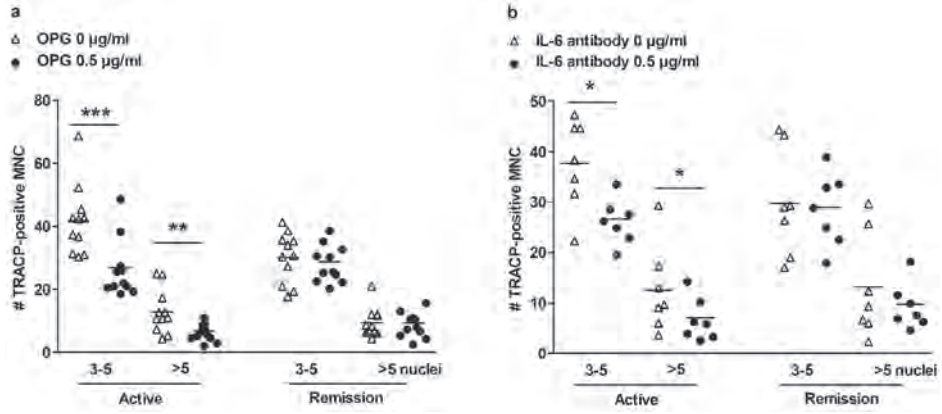

Figure 4. Inhibition of RANKL and IL-6 reduced the number of osteoclasts in active RA-serum–pretreated osteoblasts and PBMCS in co-culture. **a** OPG reduced osteoclastogenesis only in the active RA-sera pre- treated osteoblasts and PBMCS co-culture. Values are mean ± SEM from 2 independent experiments with sera obtained from 11 patients. Significant effect of OPG, ** p<0.01, *** p<0.001. **b** Inhibition of IL-6 reduced osteoclastogenesis only in active RA-sera–pretreated osteoblasts and PBMCS co-culture. Values are mean from 2 independent experiments with sera obtained from 7 patients. Significant effect of IL-6 inhibitor, * p<0.05.

DISCUSSION

We investigated whether circulating inflammatory mediators, present in serum of patients with active RA, alter osteoblast function compared with serum from the same patients in clinical remission. We demonstrated that active RA-serum inhibits osteoblast proliferation and differentiation, and affects the production of specific cytokines by osteoblasts. Active RA-sera enhanced RANKL and IL-6 gene expression in osteoblasts. Pretreatment of osteoblasts with active RA-sera stimulated the number of osteoclasts that were generated in co-culture with these osteoblasts, while addition of OPG or IL-6 inhibitory antibodies significantly reduced this number. These results indicate that the circulation of patients with active RA but not patients in remission contains factors that stimulate osteoclast formation via cytokines produced by osteoblasts.

Active RA-sera inhibited osteoblast differentiation-specific gene expression, i.e. COL1, OPN, ON, DMP1 and OCN, which is strongly supported by clinical data reported earlier by Vis and colleagues showing that serum levels of OCN and the amino-terminal propeptide of type I collagen (P1NP) are reduced in RA patients at baseline compared with the same patients after treatment with DMARDs (25). Thus *in vivo* osteoblast activity is suppressed in RA, but the mechanism of osteoblast suppression is still not fully understood. Our data indicate that sera obtained from RA patients contain factors that directly affect osteoblasts. Active RA-sera (diluted 1:10) already affected osteoblast differentiation *in vitro*, suggesting that the effects *in vivo* might even be more pronounced. Sera taken from children with polyarticular juvenile idiopathic arthritis (pJIA) also inhibit osteoblast differentiation and mineralization, while increasing osteoblast apoptosis compared with serum from healthy children (26). The factors present in the circulation of patients with RA and pJIA could be cytokines or other growth/differentiation factors. The secreted Wnt antagonist DKK1 is found at elevated levels in RA-sera, and inhibits osteoblast differentiation (27, 28). In addition, potent cytokines such as IL-6 and TNFα are found at elevated levels in RA-sera (11, 12) and high levels of IL-6 and TNFα inhibit osteoblast differentiation (29, 30). Thus it is very likely that osteoblast activity is suppressed in RA *in vivo* due to circulating cytokines present in sufficient quantities to affect the cells.

It is already well known that cytokines such as IL-6 and IL-8 are produced by osteoblasts (31). We also found that cells of the osteoblast lineage produce IL-6, IL-8, MCSF, OPG and RANKL. The elevated cytokine levels in RA-serum will be mainly derived from the inflamed synovial joints. Moreover bone is very well vascularized, and therefore it is likely that elevated levels of cytokines also reach bone, resulting in enhanced endogenous cytokine production throughout the skeleton causing a further increase of cytokine levels in the bone environment. We found that RA-sera enhanced IL-6 and RANKL gene expression in osteoblasts.

Similarly Vis and colleagues reported that RANKL and the RANKL/OPG protein ratio in RA are significantly higher at baseline compared with values obtained from the same patients after 46 weeks of treatment with DMARDs [32]. IL-6 and RANKL have potent pro-osteoclastogenic effects (13, 33, 34). We found that factors in RA-sera enhanced osteoblast production of IL-6 and RANKL, which are responsible for osteoclast formation. However, we cannot conclude that IL-6 production is "bad-to-the-bone", since bone mass is the result of a balance between osteoclast and osteoblast activity, while IL-6 is known to stimulate osteoblast differentiation in a dose-dependent manner (35). Other inflammatory factors known to enhance osteoblast activity are prostaglandin E_2 (PGE_2) and nitric oxide (NO). Thus stimulation of endogenous cytokine production does not automatically warrant the conclusion that bone loss in RA is caused by enhanced endogenous cytokine production. However, our data strongly supports that cytokine antibodies are powerful to reduce inflammation.

Our data indicate that remission of RA may help to return osteoblast activity to healthy control levels. However, sera obtained from patients with RA in remission enhanced osteoblast-mediated osteoclastogenesis to a higher extent than healthy control sera. This might be due to remaining subclinical disease activity or MTX taken by all RA patients. MTX is a known potent stimulator of osteoclast formation (36). Reduction of inflammation within the bone microenvironment favors the restoration of osteoblast function and the repair of focal bone erosions in a murine model (27). Our study appears to corroborate these findings and provides support to the concept of rapid induction of RA remission, which is critically important for the prevention of structural damage including generalized osteoporosis (37, 38).

Our study used a well defined patient group, as well as well-established co-cultures allowing direct cell-cell contact between human osteoblasts and PBMCs. We did not use recombinant RANKL for the osteoclastogenesis assay, since RANKL might mask the effect of cytokines produced by osteoblasts treated with RA-sera. The disadvantage of not using RANKL in our osteoclastogenesis experiments is that PBMCs grown without osteoblasts and RANKL do not form osteoclasts (data not shown). Therefore we were unable to test a direct effect of sera on osteoclastogenesis in our co-culture model of osteoblasts and PBMCs. In our co-culture system the PBMCs were never in touch with RA-serum since we mainly focused on the effect of RA-sera on osteoblast proliferation, differentiation, and osteoblast-mediated osteoclastogenesis.

The primary human osteoblasts used in this study are bone cells with both osteocyte properties, since they are highly mechanosensitive, and osteoblast properties, since they form mineralized nodules *in vitro*. *In vivo* osteocytes mainly communicate to osteoclasts via RANKL, and they determine serum levels of RANKL in adults (34). In the end, primary human bone cells are mostly referred to

as osteoblasts, but they also have osteocyte characteristics. So far there is no better alternative existing to study isolated human osteocytes. A limitation of our study is that the storage time of the sera was not always the same. The potency of serum decreases with time, and therefore active RA-sera stored for one more year would have lost more of its potency than shorter stored sera, resulting in, if anything, an underestimation of the effect of active RA-sera on osteoblast proliferation and differentiation. Another limitation of our study is that we were unable to analyze osteoclast activity and the role of T-cell mediated RANKL on osteoclastogenesis due to limited RA-sera volume. Future studies are needed to address the question whether RANKL produced by T cells affects osteoclastogenesis.

In conclusion, the combination of cytokines present in serum of RA patients with active disease might contribute to bone loss by directly inhibiting osteoblast proliferation and differentiation, but especially by enhancing endogenous cytokine (i.e. RANKL and IL-6) production by osteoblasts, thereby stimulating osteoclastogenesis.

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CHAPTER 5

CXCL8 and CCL20 Enhance Osteoclastogenesis via Modulation of Cytokine Production by Human Primary Osteoblasts

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Submitted for publication

ABSTRACT

Generalized osteoporosis is common in patients with inflammatory diseases, possibly because of circulating inflammatory factors that affect osteoblast and osteoclast formation and activity. Serum levels of the inflammatory factors CXCL8 and CCL20 are elevated in rheumatoid arthritis, but whether these factors affect bone metabolism is unknown. We hypothesized that CXCL8 and CCL20 decrease osteoblast proliferation and differentiation, and enhance osteoblast-mediated osteoclast formation and activity. Human primary osteoblasts were cultured with or without CXCL8 (2-200 pg/ml) or CCL20 (5-500 pg/ml) for 14 days. Osteoblast proliferation and gene expression of matrix proteins and cytokines were analyzed. Osteoclast precursors were cultured with CXCL8 (200 pg/ml) and CCL20 (500 pg/ml), or with conditioned medium (CM) from CXCL8 and CCL20-treated osteoblasts with or without IL-6 inhibitor. After 3 weeks osteoclast formation and activity were determined. CXCL8 (200 pg/ml) and CCL20 (500 pg/ml) enhanced mRNA expression of *KI67* (2.5–2.7-fold), *ALP* (1.6–1.7-fold), and IL-6 protein production (1.3–1.6-fold) by osteoblasts. CXCL8-CM enhanced the number of osteoclasts with 3-5 nuclei (1.7-fold), and with >5 nuclei (3-fold). CCL20-CM enhanced the number of osteoclasts with 3-5 nuclei (1.3-fold), and with >5 nuclei (2.8-fold). IL-6 inhibition reduced the stimulatory effect of CXCL8-CM and CCL20-CM on formation of osteoclasts. In conclusion, CXCL8 and CCL20 did not decrease osteoblast proliferation or gene expression of matrix proteins. CXCL8 and CCL20 did not directly affect osteoclastogenesis. However, CXCL8 and CCL20 enhanced osteoblast-mediated osteoclastogenesis, partly via IL-6 production, suggesting that CXCL8 and CCL20 may contribute to osteoporosis in rheumatoid arthritis by affecting bone cell communication.

KEY WORDS:
Chemokine, interleukin-6, osteoblast-to-osteoclast communication, osteooclastogenesis

INTRODUCTION

Generalized osteoporosis is common in patients with systemic inflammatory disease such as rheumatoid arthritis (RA) and Crohn's disease (1, 2). RA is a systemic, autoimmune inflammatory disease of unknown etiology characterized by chronic inflammation (3). Hallmarks of RA are local bone erosion and joint space narrowing, but extra-articular manifestations such as generalized osteoporosis are also common (1, 3). Local and generalized bone loss results from an imbalance in osteoblastic bone formation and osteoclastic bone resorption during bone remodeling. Unfortunately the underlying mechanisms of this imbalance are not fully elucidated.

The cause of bone loss during systemic inflammation is multifactorial, such as lack of physical activity, use of corticosteroids, and increased levels of inflammatory cytokines (4). Serum from patients with active RA contain circulating factors, likely cytokines and chemokines, that inhibit osteoblast proliferation and differentiation, and modulate endogenous cytokine production by osteoblasts, thereby affecting osteoclastogenesis (5). Chronic inflammation in RA enhances production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), as well as production of chemokines such as CXCL8, CXCL9, CXCL10, and CCL20 in arthritic joints (6-8). During bone remodeling, osteoblasts and osteocytes also release cytokines, e.g. receptor activator of nuclear factor-kappa B ligand (RANKL), osteoprotegerin (OPG), IL-1β, and IL-6 (9, 10). Cytokines, such as TNF-α and IL-1β, affect osteoblastic cytokine production in an autocrine manner (9, 10). TNF-α, RANKL, OPG, IL-1β, and IL-6 play a vital role in osteoclast formation and activity (9-11). Enhanced levels of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-17 in arthritic joints and in the systemic circulation do not only directly disturb the balance between osteoblastic bone formation and osteoclastic bone resorption (12, 13), but also affect osteocyte and/or osteoblast communication towards osteoclasts resulting in bone loss (14, 15). This indicates a possible role of cytokines/chemokines in bone loss during systemic inflammation.

Chemokines are small (8-12 KD) chemo-attractant cytokines, which bind G-protein–coupled receptors (16, 17). Chemokines play an important role in immunological tolerance and movement of immune cells (16). Although chemokines are involved in the pathogenesis of RA (7), their role in bone remodeling is still unclear. Indeed, elevated levels of CXCL8, CXCL9, CXCL10, and CCL20 have been demonstrated in the synovium and serum of RA patients, and receptors for these chemokines have been detected in human primary osteoblasts, i.e. the receptor for CXCL8 (CXCR1), CXCL9, CXCL10 (CXCR3), and CCL20 (CCR6) (18-22). Moreover osteoblasts from RA patients express more CCL20 and CCR6 than osteoblasts from patients with osteoarthritis or healthy

individuals. Osteocytes and mononuclear cells from RA patients also express CCL20 and CCR6 (20, 23). Thus CXCL8, CXCL9, CXCL10, and CCL20 could contribute to bone loss in inflammation, which might provide novel targets for intervention in patients with inflammatory disease, when inhibition of other cytokines such as IL-6 and TNF-α does not suffice to restore bone remodeling to normal levels.

The effect of cytokines on bone remodeling is well described, but much less is known about the effect of chemokines on bone remodeling (14). Until now, no data is available concerning the role of chemokines in localized and generalized osteoporosis in RA or other inflammatory diseases. Therefore we hypothesized that elevated levels of chemokines play a role in osteoporosis in RA. We aimed to analyze the effect of CXCL8, CXCL9, CXCL10, and CCL20 on human primary osteoblast proliferation, gene expression of matrix proteins, and osteoblast-mediated osteoclast formation and activity. Our data indicate a role for CXCL8 and CCL20 in osteoclastogenesis through their effect on osteoblasts.

MATERIALS AND METHODS

Osteoblast culture

Trabecular bone samples (surgical waste) from 4 female and 3 male donors (63.3±7.8 yrs; range 53-75 yrs) were obtained from the iliac crest during sinus floor elevation surgery using autologous bone graft. Serum C-reactive protein (CRP) levels of all donors was <2.5 mg/l, indicating no inflammatory disease. The protocol was approved by the Ethical Review Board of the VU University Medical Center and all subjects gave written informed consent.

Osteoblast cultures were established as described earlier (24). Briefly, trabecular bone fragments were placed in sterile phosphate-buffered saline (PBS), chopped into small fragments, and washed extensively with PBS. Bone fragments were then incubated with 2 mg/ml collagenase type II (Worthington, Freehold, NJ) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY)/Nutrient mixture F-12 (F-12) (DMEM/F-12, 1:1 (vol/vol)) for 2 h at 37°C in a shaking water bath to remove all adhering cells from the bone fragment surfaces. Bone fragments were then washed with medium containing 10% Fetal Clone I serum (HyClone), subdivided into equal portions, and transferred to culture flasks (Nunc, Roskilde, Denmark). To obtain outgrowth of bone cells, bone fragments were cultured in DMEM/F-12 supplemented with 10% Fetal Clone I serum, 100 U/ml penicillin (Sigma, Hamburg, Germany), 100 µg/ml streptomycin sulfate (Gibco), 50 µg/ml gentamicin (Gibco), 1.25 µg/ml fungizone (Gibco), and 100 µg/ml ascorbate (Merck, Darmstadt, Germany) at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was refreshed twice a week until cells reached confluence. After reaching confluency, cells were trypsinized and seeded at $1x10^4$ cells/well of 24-well culture plates (Greiner Bio-One, Frickenhausen, Germany), incubated overnight, and treated with or without chemokines as described below.

Flow cytometric analysis of functional chemokine receptors on osteoblasts

Osteoblasts obtained from bone biopsies of 3 donors (two females: 53 and 58 yrs, and one male: 66 yrs) were used to analyze the expression of chemokine receptors. Osteoblasts were harvested at 80-90% confluency. Cells were resuspended in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin and 0.1% sodium azide for FACS analysis. For cell surface receptor antigen detection, cells were incubated with fluoro-isothiocyanate (FITC) or phycoerythrin (PE)-conjugated antibodies. $5x10^4$ cells were incubated for 30 min at 4°C with human antibodies CD44-PE (G44-26, IgG2b), CD44-FITC (G44-26, IgG2b), CCR6-PE (11A9, IgG1), CXCR1-PE (5A12, IgG1), CXCR3-PE (1C6/cxcr3, IgG1). $CD44^+$ cells were gated using CD44-PE and CD44-FITC antibody. Chemokine receptor expression was further analyzed in the $CD44^+$ cell population on a FACScan with Cell Quest software (Becton Dickinson Immunocytometry Systems, Mountain View,

CA). All antibodies used were from BD Pharming (San Diego, CA). To analyze the functionality of CXCR1 and CCR6, osteoblasts were cultured for 48 h with the chemokine ligand CXCL8 (20 pg/ml) for CXCR1 and CCL20 (50 pg/ml) for CCR6. Chemokine-treated osteoblasts were then analyzed again for CXCR1 and CCR6 expression.

Chemokine treatment

Osteoblasts obtained from bone biopsies from 4 different donors (two females: 59 and 61 yrs, and two males: 71 and 75 yrs) were used to analyze the effect of CXCL8 and/or CCL20, and TNF-α on osteoblast proliferation and differentiation, and osteoblast-to osteoclast communication. Osteoblasts seeded in 24 well-culture plates were incubated overnight and treated with or without recombinant human CXCL8 (2, 20, 200 pg/ml; Sigma), CCL20 (5, 50, 500 pg/ml; Sigma), a combination of CXCL8 (20 ng/ml) + CCL20 (50 ng/ml), or TNF-α (100 ng/ml; Sigma) for 14 days. The effect of CXCL8, CCL20, CXCL8+CCL20, or TNF-α on osteoblast proliferation and differentiation was analyzed. Chemokine concentrations tested were chosen based on RA-serum concentrations. Concentrations which were 10 times lower or higher than the levels reported in RA-serum were also tested (7, 8). TNF-α was used as positive control. Chemokine treatment was performed for 14 days to mimic *in vivo* chronic inflammation as in RA. CM from osteoblasts cultured for 14 days with control medium (control-CM), CXCL8 at 2, 20, and 200 pg/ml (CXCL8-CM), CCL20 at 5, 50, and 500 pg/ml (CCL20-CM), CXCL8 + CCL20 (CXCL8+CCL20-CM) and TNF-α (TNF-α-CM) were collected.

Osteoblast proliferation

Proliferation of osteoblasts was tested in cells seeded at $2.5x10^3$ cells/well of 96-well culture plates (Greiner Bio-One). The following day, medium was replaced by fresh medium with or without CXCL8, CCL20, CXCL8+CCL20, or TNF-α. Cell proliferation was determined after 3, 5, and 7 days of culture using a Cell Proliferation Kit II (XTT; Roche, Mannhelm, Germany).

The total DNA content of the cell layer was quantified using a Cyquant Cell Proliferation Assay (Molecular Probes, Eugene, OR).

Procollagen type 1 amino-terminal propeptide (P1NP) and alkaline phosphatase (ALP) activity

P1NP in CM and ALP in cell lysate of osteoblast cultures were measured as described earlier (25).

RNA isolation and real-time RT-PCR

Total RNA of primary osteoblasts was isolated using an RNeasy® Micro kit with an on-column DNase I digestion (Qiagen, Basel, Switzerland). Total RNA concentrations were measured with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA synthesis was performed in a thermocycler GeneAmp® PCR System 9700 PE (Applied Biosystems, Foster City, CA), using a SuperScript® VILO™ cDNA Synthesis Kit (LifeTechnologies, Inchinnan, UK), with 0.1 µg of total RNA in 20 µl reaction mixture consisting of VILO Reaction Mix and SuperScript Enzyme Mix.

Real-time PCR reactions were performed using 2.5 µl cDNA and SYBR® Green Supermix (Roche Laboratories, Indianapolis, IN) in a LightCycler® (Roche Diagnostics). In each PCR run, the reaction mixture without cDNA was used as a negative control. For quantitative real-time PCR, the values of relative target gene expression were normalized for relative mean of *YWHAZ* and *HPRT* housekeeping gene expression. Real-time PCR was used to assess expression of the following genes: *KI67*, collagen 1 (*COL1*), *ALP*, osteopontin (*OPN*), osteocalcin (*OCN*), macrophage colony stimulating factor (*MCSF*), *RANKL*, *OPG*, *IL-1β*, *IL-6*, *IL-17*, *CXCL8*, *CCL20*, and cysteine rich protein 61 (*CYR61*). All primers used were from LifeTechnologies. The primer sequences are listed in Table 1.

IL-6 protein quantification

CM was collected from osteoblasts after 14 days of culture in the presence or absence of chemokines, and IL-6 protein was quantified using a PeliKine^{TM} human IL-6 ELISA kit (Sanquin Blood Supply, Amsterdam, Netherlands).

Osteoclastogenesis

Peripheral blood mononuclear cells (PBMCs) were isolated from a buffy coat (Sanquin) as described previously (26). Buffy coats were obtained from blood donated by healthy blood donors at Sanquin Blood Supply, Amsterdam, The Netherlands. PBMCs were seeded at $5x10^5$ cells/well of 96 well plates or on bovine bone slices in DMEM containing 10% FCS, antibiotics, and control-CM, CXCL8-CM from 200 pg/ml CXCL8 treatment, CCL20-CM from 500 pg/ml CCL20 treatment, CXCL8+CCL20-CM, and TNF-α-CM (ratio DMEM:CM = 1:1 (v/v)). Twenty-five ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN) was added to the cells from day 1 to day 3. Ten ng/ml M-CSF and 4 ng/ml human RANKL (Peprotech, London, UK) were added from day 3 to day 21. To control-CM added osteoclastogenesis culture, 0.15 µg/ml human IL-6 antibody (Clone #6708, R&D Systems) was added and IgG isotype control (Clone #11711, R&D Systems) was used as control for IL-6 antibody. PBMCs were also cultured with DMEM containing 10% FCS, antibiotics, and either CXCL8 (200 pg/ml) or CCL20 (500 pg/ml). After 3 weeks, cells were fixed in 4% formaldehyde, and stained for tartrate-resistant acid phosphatase (TRACP; Sigma). Nuclei were visualized by 4',6-diamidino-2-

phenylindole (DAPI) staining. Osteoclastogenesis was assessed by counting the number of TRACP-positive osteoclasts containing >3 nuclei per cell on 10 pre-determined microscopic fields in the each well.

Osteoclastic bone resorption

To quantify osteoclast activity, resorption pits in bone slices were visualized and counted as described earlier (27). The resorbed area was measured using Image Pro-Plus Software (Media Cybernetics, Silver Spring, MD) and expressed as percentage of total bone surface area. The percentage of bone resorption was expressed per number of TRACP-positive multinucleated cells.

Statistical analysis

Data on *KI67* gene expression, TRACP-positive multinucleated osteoclast number, and osteoclastic bone resorption was expressed as mean±SEM. Data on expression of other genes analyzed and IL-6 protein production were expressed as median with 5-95 percentile range of the treatment-over-control ratio. Differences in gene expression and IL-6 protein production between chemokine-treated and untreated control cultures were tested using the Wilcoxon Signed Rank test. The effect of control-CM or chemokine-CM on osteoclastic bone resorption, and the effect of control-CM or chemokine-CM and CM+IL-6 inhibitor on osteoclast formation was tested using ANOVA followed by Bonferroni's Multiple Comparison test. Differences were considered significant if $p<0.05$. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Table 1. Primers used in the real-time PCR assay.

Gene		Oligonucleotide sequence	Amplicon length (bp)
YWHAZ	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'	
HPRT1	Forward	5' GCTGACCTGCTGGATTACAT 3'	260
	Reverse	5' CTTGCGACCTTGACCATCT 3'	
KI67	Forward	5' GGTGGGCACCTAAGACCTGAA 3'	235
	Reverse	5' TCCTAGGACTAGGAGCTGGAG 3'	
ALP	Forward	5' AGGGACATTGACGTGATCAT 3'	242
	Reverse	5' CCTGGCTCGAAGAGACC 3'	
COL1	Forward	5' TCCAACGAGATCGAGATCC 3'	191
	Reverse	5' AAGCCGAATTCCTGGTCT 3'	
OPN	Forward	5' TTCCAAGTAAGTCCAACGAAAG 3'	181
	Reverse	5' GTGACCAGTTCATCAGATTCAT 3'	
OCN	Forward	5' AGCCACCGAGACACCATGAGA 3'	288
	Reverse	5' CTCCTGAAAGCCGATGTGGTC 3'	
IL-6	Forward	5' ACAGCCACTCACCTCTTCA 3'	207
	Reverse	5' ACCAGGCAAGTCTCCTCAT 3'	
IL-1B	Forward	5' TGGAGCAACAAGTGGTGTTCT 3'	270
	Reverse	5' GAGAGGTGCTGATGTACCAGTT 3'	
RANKL	Forward	5' CATCCCATCTGGTTCCCATAA 3'	60
	Reverse	5'GCCCAACCCCGATCATG 3'	
M-CSF	Forward	5' CCGAGGAGGTGTCGGAGTAC 3'	100
	Reverse	5' AATTTGGCACGAGGTCTCCAT 3'	
OPG	Forward	5'TGGAATAGATGTTACCCTGTGTG 3'	298
	Reverse	5' GCTGCTCGAAGGTGAGGTTA 3'	
TNF-α	Forward	5' AGAGGGCCTGTACCTCATCT 3'	315
	Reverse	5' AGGGCAATGATCCCAAAGTAG 3'	
CYR61	Forward	5' CAACCCTTTACAAGGCCAGA 3'	206
	Reverse	5' TGGTCTTGCTGCATTTCTTG 3'	
CCL20	Forward	5' TGATGTCAGTGCTGCTACTC 3'	142
	Reverse	5' ATGTCACAGCCTTCATTGGC 3'	
IL17	Forward	5' CTACAACCGATCCACCTCAC 3'	255
	Reverse	5' ACAATCGGGGTGACACAGGT 3'	
CXCL8	Forward	5' TCTGCAGCTCTGTGTGAAG 3'	147
	Reverse	5' TGTGTTGGCGCAGTGTGG 3'	

RESULTS

Osteoblasts expressed CXCL8 and CCL20 receptors

Osteoblasts were highly positive for chemokine receptor CXCR1 (72.4±7.7%, mean±SEM), and to a lesser extent for CCR6 (15.6±8.2%; Fig. 1). Osteoblasts did not express CXCR3. After 48 h of culture with chemokines, CXCR1 receptor expression decreased by 70% and CCR6 expression by 64% (Table 2). Since osteoblasts did not express the chemokine receptor CXCR3 for CXCL9 and CXCL10, these chemokines were not used for further study.

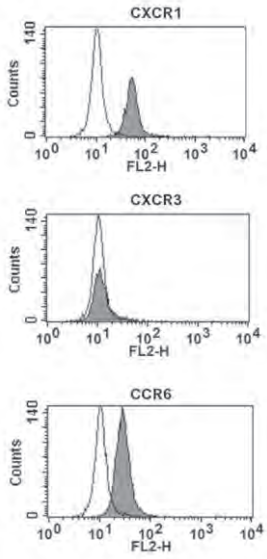

Figure 1. Osteoblasts expressed CXCR1 and CCR6 receptors. Flow cytometric analysis of CXCR1, CXCR3 and CCR6 receptor expression by primary bone cells. Open histogram: isotype control; shaded histogram: chemokine receptor antibody.

Table 2. Functional chemokine receptor expression in human primary bone cells after 48 h of culture with or without CXCL8 or CCL20.

	% cells expressing receptor	
	without CXCL8 or CCL20	with CXCL8 or CCL20
CXCR1	72.4 ± 7.7	21.2 ± 6.4
CCR6	15.0 ± 8.2	5.6 ± 1.8

Values are mean ± SD of the percent of human primary bone cells expression CXCR1 or CCR6. n=3 experiments, using cells obtained from independent donors.

CXCL8 and CCL20 did not inhibit proliferation nor gene expression of matrix proteins in osteoblasts

CXCL8 (200 pg/ml) increased *KI67* gene expression by 2.7-fold at day 8 and 1.6-fold at day 14 (Fig. 2A). CCL20 (500 pg/ml) increased *KI67* expression by 2.4 fold at day 6 and 2.5-fold at day 8 (Fig. 2B). The other CXCL8 and CCL20 concentrations tested did not affect *KI67* expression. The combination of CXCL8+CCL20 (20 pg/ml+50 pg/ml) enhanced *KI67* expression by 3.7-fold at day 8 (Fig. 2C). TNF-α enhanced *KI67* expression by 2.3, 2.6, and 1.6-fold at day 6, 8, and 14 respectively (Fig. 2D). CXCL8 and/or CCL20 treatment did not affect osteoblast proliferation as measured by the XTT assay, nor the total DNA content (data not shown).

CXCL8 (200 pg/ml) increased gene expression of differentiation marker *ALP* by 1.7-fold, and *COL1* by 1.5-fold at day 14 (Fig. 2E). CCL20 (500 pg/ml) increased *ALP* expression by 1.6-fold (Fig. 2D). CXCL8 (200 pg/ml) and CCL20 (500 pg/ml) did not affect gene expression of the matrix proteins *OPN* and *OCN*. Neither CXCL8 at 2 or 20 pg/ml, CCL20 at 5 or 50 pg/ml, nor TNF-α (100 ng/ml) or CXCL8+CCL20 (20 pg/ml+50 pg/ml) did affect expression of COL1*, OPN, or OCN*. P1NP formation and ALP activity was not changed by chemokines or TNF-α (data not shown).

CXCL8 and CCL20 enhanced *IL-6* gene expression and IL-6 protein production by osteoblasts

CXCL8 (200 pg/ml) increased gene expression of MCSF by 1.3-fold and *IL-6* by 1.4-fold at day 14 (Fig. 3A). CCL20 (500 pg/ml) increased *IL-6* expression by 1.6-fold (Fig. 3B). The combination of CXCL8+CCL20 (20 pg/ml+50 pg/ml) increased expression of *IL-6* by 1.7-fold, *OPG* by 1.4-fold, and *CYR61* by 1.7-fold (Fig. 3C). TNF-α (100 ng/ml) increased expression of *MCSF* by 2.8-fold, *IL-6* by 64-fold, *OPG* by 2.1-fold, and CXCL8 by 836-fold (Fig. 3D). Since only IL-6 gene expression was enhanced by CXCL8 (200 pg/ml), CCL20 (500 pg/ml), CXCL8+CCL20 (20 pg/ml+50 pg/ml), and TNF-α (100 ng/ml) treatment in osteoblasts, we measured IL-6 protein production in the CM from osteoblasts. CXCL8 (200 pg/ml), CCL20 (500 pg/ml), CXCL8+CCL20 (20 pg/ml+50 pg/ml), and TNF-α (100 ng/ml) increased IL-6 production by 1.3, 1.6, 2.2, and 29-fold respectively compared to controls (Fig 3E). The mean IL-6 concentration in control CM was 148 pg/ml. Gene expression of *TNF-α, RANKL, IL-1β, CCL20*, and *IL-17* was below the detection limit (ct value >38).

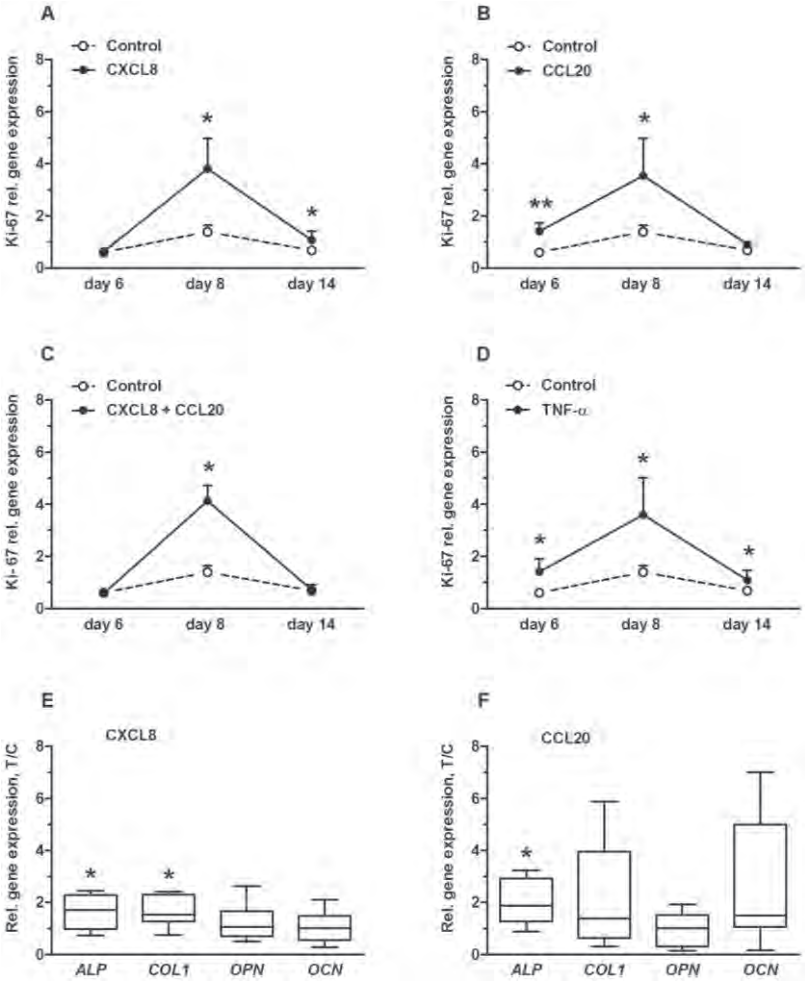

Figure 2. CXCL8 (2, 20, and 200 pg/ml) and CCL20 (5, 50, and 500 pg/ml) enhanced Ki-67 gene expression, but only 200 pg/ml of CXCL8 and 500 pg/ml of CCL20 enhanced early osteogenic marker gene expression in osteoblasts. **(A)** CXCL8 (200 pg/ml) enhanced Ki-67 expression (day 8,14). **(B)** CCL20 (500 pg/ml) enhanced ki-67 expression (day 6,8). **(C)** CXCL8+CCL20 (20 pg/ml+50 pg/ml) enhanced ki-67 expression (day 8). **(D)** TNF-α (100 ng/ml) enhanced Ki-67 expression (day 6,8,14). **(E)** CXCL8 (200 pg/ml) enhanced ALP and COL1 expression (day 14). **(F)** CCL20 (500 pg/ml) enhanced ALP expression (day 14). Values are median with 5-95 percentile range of treatment-over-control ratios from 3 experiments, n=9. Significant effect of chemokines or TNF-α, *p<0.05, **p<0.01.

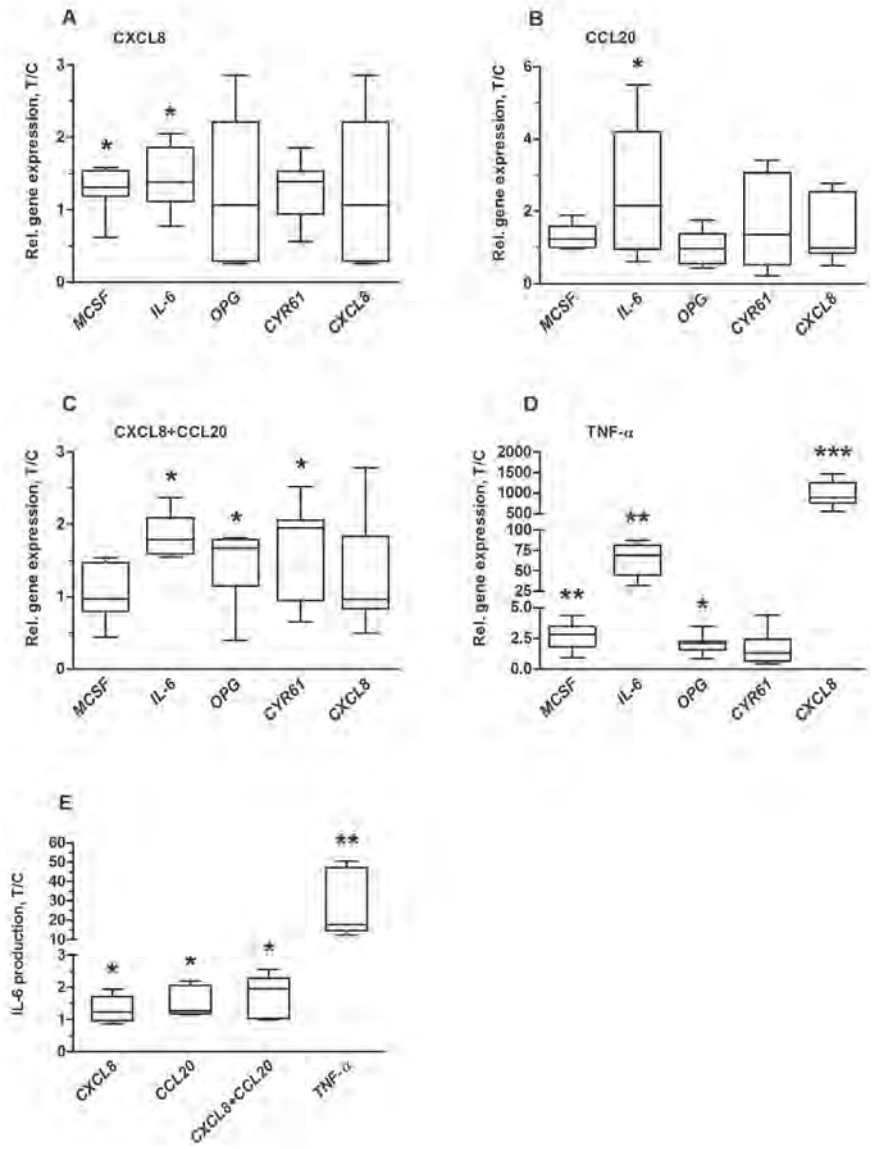

Figure 3. CXCL8 (200 pg/ml) and CCL20 (500 pg/ml) affected cytokine gene expression by osteoblasts at day 14. **(A)** CXCL8 (200 pg/ml) enhanced MCSF and IL-6 gene expression. **(B)** CCL20 (500 pg/ml) enhanced IL-6 gene expression. **(C)** CXCL8+CCL20 (20 pg/ml+50 pg/ml) enhanced IL-6, OPG, and CYR61 gene expression. **(D)** TNF-α (100 ng/ml) enhanced MCSF, IL-6, and OPG gene expression. **(E)** CXCL8 (200 pg/ml), CCL20 (500 pg/ml), CXCL8+CCL20 (20 pg/ml+50 pg/ml), and TNF-α (100 ng/ml) enhanced IL-6 production by osteoblasts. Values are median with 5-95 percentile range of treatment-over-control ratios from 3 experiments, n=9. Significant effect of chemokines and TNF-α, *p<0.05, **p<0.01.

CXCL8 and CCL20 enhanced osteoclastogenesis partly via IL-6 production by osteoblasts

CXCL8 and CCL20 did not directly affect osteoclast formation (Fig. 4A). In contrast, CM from osteoblasts cultured for 14 days increased osteoclast number by 1.5-fold (Fig. 4B). CXCL8-CM increased the number of osteoclasts with 3-5 nuclei by 1.7-fold, and with >5 nuclei by 3.0-fold (Fig. 4C). Inhibition of IL-6 nullified the stimulatory effect of IL-6 on the formation of osteoclasts with 3-5 nuclei, and reduced the formation of osteoclasts with >5 nuclei from 3-fold to 1.3-fold (Fig. 4C). CCL20-CM increased the number of osteoclasts with 3-5 nuclei by 1.3-fold, and with >5 nuclei by 2.8-fold (Fig. 4D). IL-6 inhibition nullified the stimulatory effect of IL-6 on the formation of osteoclasts with 3-5 nuclei, and reduced the formation of osteoclasts with >5 nuclei from 2.8-fold to 1.3-fold (Fig. 4D). CXCL8+CCL20-CM increased the number of osteoclasts with 3-5 nuclei by 1.3-fold, and with >5 nuclei by 2.5-fold (Fig. 4E). IL-6 inhibition nullified the stimulatory effect of IL-6 on the formation of osteoclasts with 3-5 nuclei, and reduced the formation of osteoclasts with >5 nuclei from 2.5-fold to 1.3-fold (Fig. 4E). TNF-α-CM increased the number of osteoclasts with >5 nuclei by 3.7-fold, while IL-6 inhibition reduced the formation of osteoclasts with 3-5 nuclei from 3.7-fold to 1.4-fold (Fig. 4F). Addition of IgG isotype control for IL-6 did not affect the number of osteoclasts that developed in control-CM added cultures (data not shown).

CCL20-CM enhanced osteoclastic bone resorption

Osteoclasts formed resorption pits on bovine cortical bone slices (Fig. 5A,B). CCL20-CM enhanced osteoclastic bone resorption by 2.2-fold in comparison to control-CM (Fig. 5C). CXCL8-CM, CXCL8+CCL20-CM, and TNF-α-CM did not affect osteoclastic bone resorption (Fig. 5C).

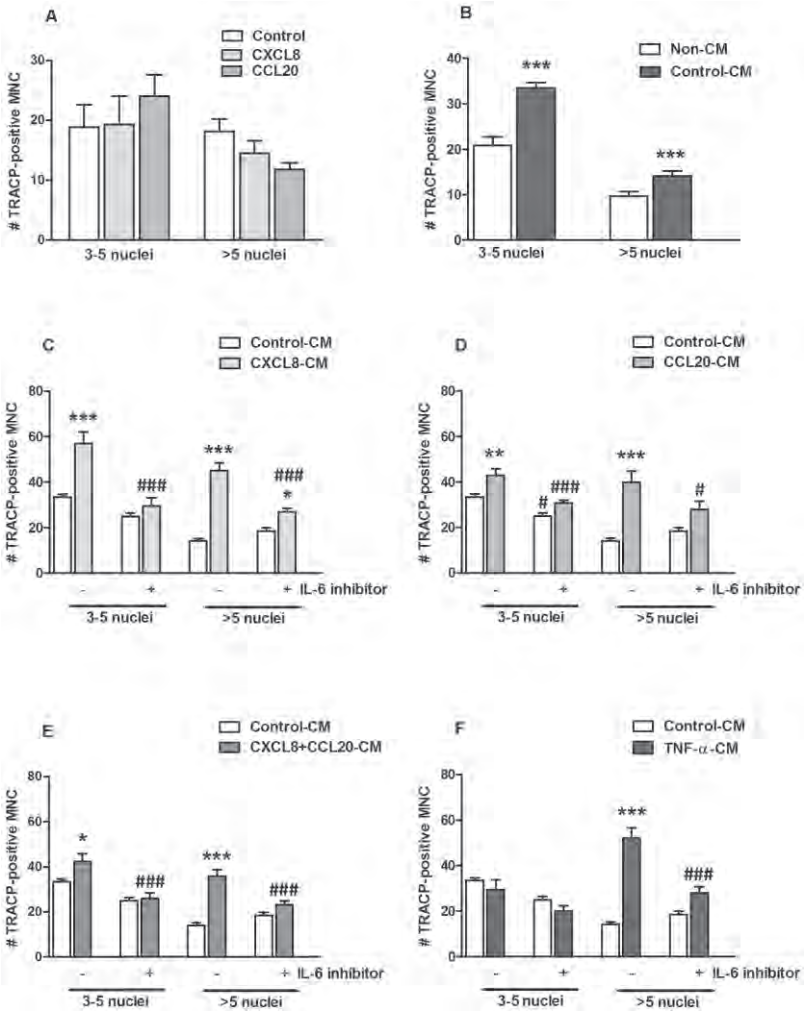

Figure 4. Effect of CXCL8 (200 pg/ml), CCL20 (500 pg/ml), CXCL8-CM, and CCL20-CM on osteoclast formation. (**A)** CXCL8 (200 pg/ml) and CCL20 (500 pg/ml) did not affect TRACP-positive multinucleated cell ($TRACP^+$ MNC) number. **(B)** CM from osteoblasts cultured without chemokines enhanced $TRACP^+$ MNC number. **(C)** CXCL8-CM enhanced osteoclastogenesis. IL-6 inhibition reduced this effect. **(D)** CCL20-CM enhanced osteoclastogenesis. IL-6 inhibition reduced this effect. **(E)** CM from osteoblasts cultured with CXCL8+CCL20 (20 pg/ml+50 pg/ml) enhanced osteoclastogenesis. IL-6 inhibition reduced this effect. **(F)** TNF-α-CM enhanced osteoclastogenesis (osteoclasts with >5 nuclei). IL-6 inhibition reduced this effect. Values are mean±SEM from 3 experiments, n=9. Significant effect of chemokine, control-CM, and chemokine-CM, *p<0.05, **p<0.01, ***p<0.001. Significant effect of IL-6 inhibitor, $^{\#}p<0.05$, $^{\#\#\#}p<0.001$.

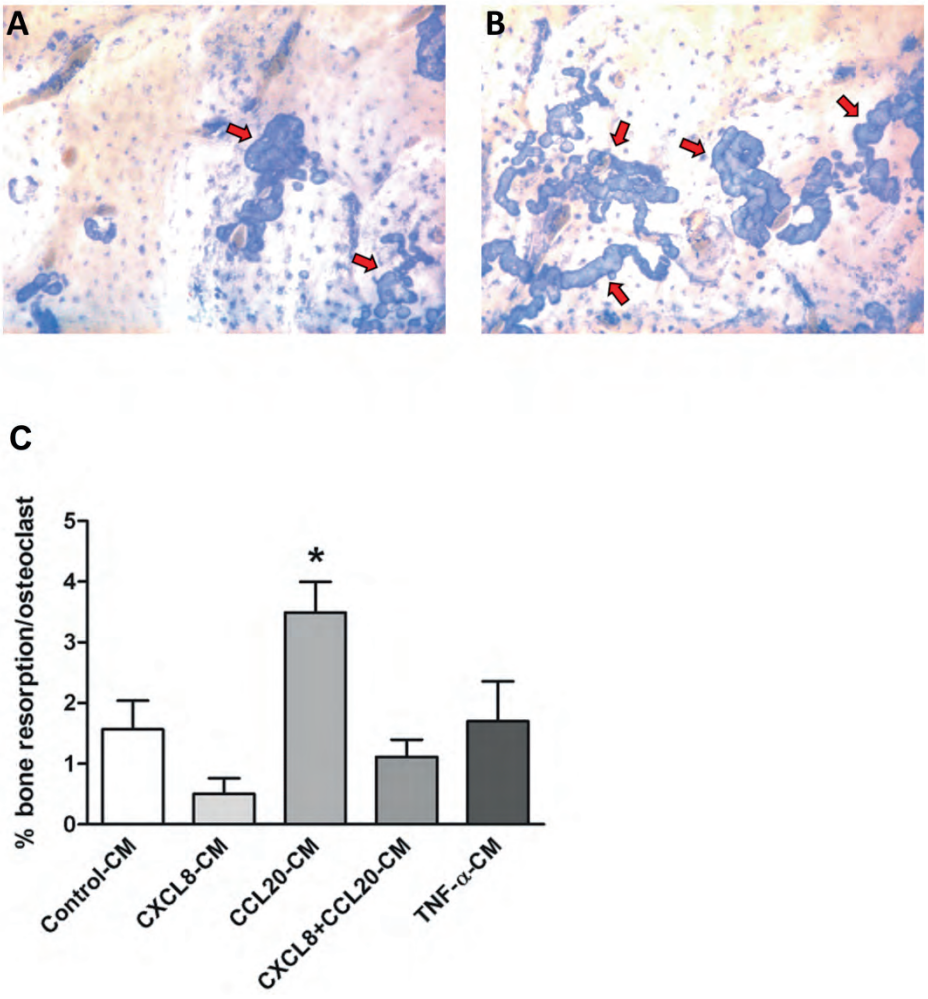

Figure 5. Effect of CM from osteoblasts cultured with CXCL8 (200 pg/ml) or CCL20 (500 pg/ml) on osteoclastic bone resorption. **(A)** Resorption pits (blue area; arrows) on a bone slice after 21 days of culture of PBMCs in the presence of control-CM. CXCL8-CM, CXCL8+CCL20-CM, and TNF-α-CM resulted in resorption pit formation, similar to those formed in the presence of control-CM (data not shown). **(B)** Resorption pits (blue area; arrows) on a bone slice after 21 days of culture of PBMCs in the presence of CCL20-CM. **(C)** CCL20 (500 pg/ml)-CM enhanced osteoclastic bone resorption compared to control-CM. CXCL8 (200 pg/ml)-CM, CXCL8 (20 pg/ml) +CCL20 (50 pg/ml)-CM, and TNF-α (100 ng/ml)-CM did not affect osteoclastic bone resorption. Values are mean±SEM from 3 experiments, n=9. Significant effect of CM, *$p<0.05$.

DISCUSSION

In this study we analyzed whether chemokines potentially play a role in the emergence of osteoporosis in inflammatory diseases by studying their effect on osteoblast function and osteoblast-mediated osteoclast formation and activity. We found that CXCL8 and CCL20 did not inhibit osteoblast proliferation nor gene expression of the main matrix proteins *COL1*, *OPN*, and *OCN*. CXCL8 and CCL20 enhanced osteoblast-mediated osteoclastogenesis, partly via IL-6 production, suggesting that CXCL8 and CCL20 may contribute to generalized osteoporosis in RA by affecting bone cell communication.

The osteoblastic nature of human primary osteoblasts, as well as their osteocyte-like behavior has been shown earlier (28-31). We found that these cells express functional chemokine receptors CXCR1 and CCR6, but not CXCR3, which is the receptor for CXCL9 and CXCL10. Lisignoli and colleagues reported that osteoblasts express functional CXCR3 (18). This discrepancy in CXCR3 expression might be related to differences in anatomical location of the bone biopsies; we used biopsies from the iliac crest, and Lisignoli and colleagues from the tibial plateau (18).

Both CXCL8 and CCL20 enhanced gene expression of the cell proliferation marker *KI67* in osteoblasts. However we could not detect an effect of chemokines on osteoblast proliferation using the XTT assay or by measuring the total DNA content, which is in contrast with data published by others showing that CCL20 enhances proliferation of osteoblasts from RA patients (19). This difference might be due to the fact that we obtained cells from patients without any signs of systemic inflammation, while Lisignoli and colleagues obtained cells from RA patients (19). Our findings indicate that CXCL8 and CCL20 do not affect osteoblast proliferation.

Since proinflammatory cytokines inhibit osteoblast function (14, 32-34) we expected that CXCL8 or CCL20 would also inhibit osteoblast function. However we found that CXCL8 and CCL20 enhanced gene expression of some early osteogenic differentiation-related markers but did not affect P1NP production, ALP activity, nor gene expression of late osteogenic differentiation-related markers. CXCL8 and CCL20 increased *ALP* and *COL1* gene expression, but not P1NP and ALP activity, which might be due to altered post-transcriptional processing. In any case, our data suggests that CXCL8 and CCL20 do not negatively affect osteoblast function *in vitro*.

CXCL8 and CCL20 enhanced *IL-6* gene expression and protein production by osteoblasts. CXCL8 and CCL20 did not directly affect osteoclastogenesis, which is in accordance with findings by others (19, 20). Here we show that CM from osteoblasts cultured for 14 days (control-CM; without chemokines) enhanced osteoclastogenesis *in vitro*, indicating that osteoblasts produce factors essential for

osteoclast formation. Furthermore, our study reveals that CXCL8-CM, CCL20-CM, and TNF-α-CM enhanced osteoclastogenesis. CCL20-CM, but not CXCL8-CM, enhanced osteoclast activity. Osteoclast activity is not only regulated by the number of osteoclasts and tartrate-resistant acid phosphatase present in osteoclasts, but also by a number of other regulators such as cathepsin K, lysophosphatidic acid receptor type 1 (LPA1), lysosome associated membrane protein-2, and chloride channels CIC3 and CIC7 (35-38). Osteoclast activity regulators might present differently in osteoclasts formed in the presence of CXCL8-CM compared with CCL20-CM, which might cause the difference in osteoclastic bone resorbing activity. We also found that CXCL8 and CCL20 enhanced *IL-6* gene expression and protein production by osteoblasts. Moreover IL-6 production was strongly enhanced by TNF-α treatment; a similar finding has been reported by Chaudhary et al. (9). Inhibition of IL-6 robustly reduced the effect of CXCL8-CM, CCL20-CM, and TNF-α-CM on osteoclastogenesis. CCL20 has been suggested to play a role in the pathogenesis of rheumatoid arthritis (39). IL-6 is one of the most potent stimulators of osteoclastic bone resorption and central to the pathogenesis of generalized osteoporosis in RA (40-42). This corroborates with our data showing a stimulatory effect of CXCL8 and CCL20 on IL-6 production by osteoblasts, which enhanced osteoclastogenesis. CXCL8 and CCL20 might not only affect IL-6 production, but also the production of a whole cocktail of factors by osteoblasts. Therefore it is unlikely that IL-6 inhibition alone will completely block the effect of CXCL8 and/or CCL20 on osteoblast-mediated osteoclastogenesis. RANK, RANKL, and OPG are known key molecules involved in osteoclast formation and function (43). In this study, RANKL gene expression by osteoblasts was below the detection limit, and therefore we did not test the effect of RANKL and OPG signaling molecules produced by osteoblasts on osteoclast formation. Our model uses exogenous recombinant RANKL to allow osteoclast formation, since osteoclast formation does not occur in the absence of RANKL, which created further limitation to analyze the role of RANKL and OPG in osteoclastogenesis. Detailed information on our model is provided in Figure 6. Based on our findings, we created a pathophysiological model illustrating how CXCL8 and CCL20 might influence bone remodeling in inflammatory conditions and contribute to osteoporosis (Figure 7).

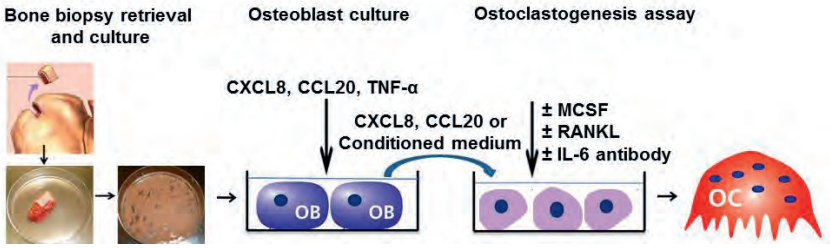

Figure 6. Schematic representation of experimental set up. Human primary osteoblasts were cultured with CXCL8 (200pg/ml), CCL20 (500 pg/ml), CXCL8+CCL20 (20 pg/ml+50 pg/ml), or TNF-α (100 ng/ml) for 14 days. Conditioned medium from all cultures (control-CM, CXCL8-CM, CCL20-CM, CXCL8+CCL20-CM, TNF- α-CM) were colleted. Osteoclast precursors were cultured with these conditioned medium and fresh medium in 1:1 (vol:vol) in presence of MCSF and RANKL±IL-6 antibody for 21 days. Osteoclasts were stained by TRACP staining and counted.

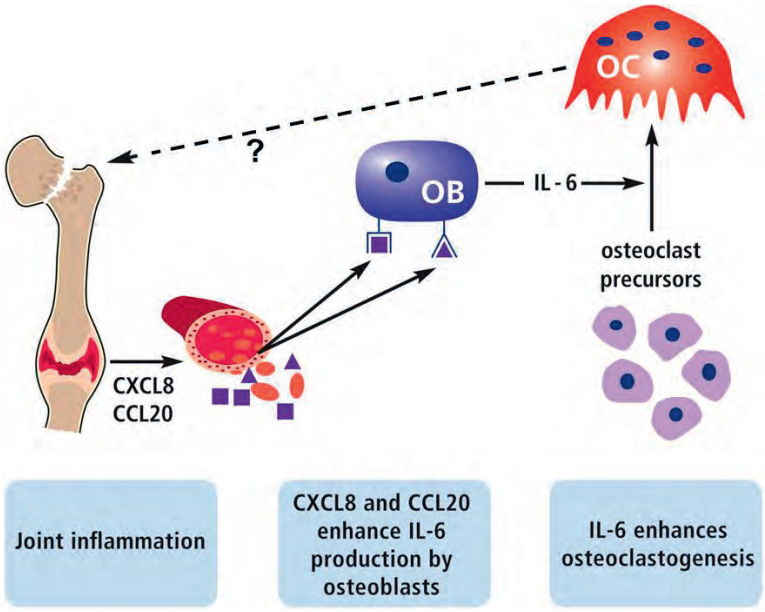

Figure 7. Pathophysiological model. Illustration of how CXCL8 and CCL20 might influence bone remodeling during systemic inflammation such as RA and might contribute to osteoporosis.

CXCL8 and CCL20 are abundantly present in synovial tissue, synovial fluid, and serum in RA, and their levels correlate with disease activity (8, 20, 44, 45). Blockade of CXCL8 reduces inflammation in a murine RA model (46). A CXCR1 antagonist has been shown to decrease clinical disease scores in a murine collagen-induced arthritis model (47). A polymorphism of the CCR6 gene has been associated with RA susceptibility (48). Moreover CCL20 has been suggested as an emerging player in the pathogenesis of rheumatoid arthritis (39). Our study provides insight in the mechanism of action of CXCL8 and CCL20 with regard to the regulation of bone metabolism. Therefore either CXCL8 and/or CCL20 might be new targets to prevent bone loss in inflammatory diseases such as RA. Future *in vivo* studies could further elucidate the degree and mechanism of the effect of CXCL8 and CCL20 on bone loss during systemic inflammation. A limitation of this

study might be the relatively low number of patients included. Statistical significance between groups was not easily obtained due to the fairly large data variation, probably due to donor variation.

In conclusion, our results indicate that CXCL8 and CCL20 did not significantly inhibit osteoblast proliferation and function, nor directly enhanced osteoclastogenesis. However, CXCL8 and CCL20 strongly enhanced osteoblast-mediated osteoclastogenesis, which seems partially mediated by CXCL8 and CCL20-induced IL-6 production by osteoblasts. Based on these findings, we speculate that CXCL8 and CCL20 may play a role in generalized osteoporosis during systemic inflammation in which serum levels of CXCL8 and CCL20 are elevated.

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CHAPTER 6

Mechanical Loading Reduces Inflammation-Induced Human Osteocyte-to-Osteoclast Communication

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ABSTRACT

Multiple factors contribute to bone loss in inflammatory diseases such as rheumatoid arthritis (RA), but circulating inflammatory factors and immobilization play a crucial role. Mechanical loading prevents bone loss in the general population, but the effects of mechanical loading in patients with RA are less clear. Therefore we aimed to investigate whether mechanical stimuli reverse the stimulatory effect of RA-serum on osteocyte-to-osteoclast communication. Human primary osteocytes were pretreated with 10% RA-serum or healthy control-serum for 7 days, followed by 1h±mechanical loading by pulsating fluid flow (PFF). Nitric oxide (NO) and prostaglandin E_2 (PGE_2) were measured in the medium. Receptor-activator of nuclear-factor-kappaB ligand (RANKL), osteoprotegerin (OPG), interleukin-6 (IL-6), cyclooxygenase-2 (COX2), matrix-extracellular phosphoglycoprotein (MEPE), cysteine-rich protein 61 (CYR61), and SOST gene expression was quantified by qPCR. Osteoclast precursors were cultured with PFF-conditioned medium (PFF-CM) or static-conditioned medium (stat-CM), and osteoclast formation was assessed. RA-serum alone did not affect IL-6, CYR61, COX2, MEPE, or SOST gene expression in osteocytes. However RA-serum enhanced the RANKL/OPG expression ratio by 3.4-fold, while PFF nullified this effect. PFF enhanced NO production to the same extent in control-serum (2.6-3.5 fold) and RA-serum–pretreated (2.7-3.6 fold) osteocytes. Stat-CM from RA-serum–pretreated osteocytes enhanced osteoclastogenesis compared with stat-CM from control-serum–pretreated osteocytes, while PFF nullified this effect. In conclusion, RA-serum, containing inflammatory factors, did not alter the intrinsic capacity of osteocytes to sense mechanical stimuli, but upregulated osteocyte-to-osteoclast communication. Mechanical loading nullified this upregulation, suggesting that mechanical stimuli could contribute to the prevention of osteoporosis in inflammatory disease.

KEYWORDS:
Rheumatoid arthritis, generalized osteoporosis, inflammatory cytokines, pulsating fluid flow, osteoclastogenesis

INTRODUCTION

Many patients with chronic inflammatory disease such as rheumatoid arthritis (RA) suffer from generalized osteoporosis (1, 2). The cause of bone loss during inflammation is multifactorial and includes reduced physical activity, use of corticosteroids, and increased levels of inflammatory cytokines (2-4). Altered levels of growth factors, growth factor antagonists, and inflammatory cytokines such as IL-1β, IL-6, and TNFα are present in synovial fluid and serum from RA patients (5-7). These factors present in RA-serum easily reach the bone and affect the formation and function of osteoblasts, osteoclasts, and osteocytes as well as the communication between these bone cells (7-9). Circulating inflammatory cytokines in patients with inflammatory disease likely play an important role in bone homeostasis, since individual cytokines such as IL-1β enhance osteocyte-mediated osteoclastogenesis (10), and serum from patients with active RA inhibits osteoblast proliferation and differentiation, and enhances osteoblast-mediated osteoclastogenesis (8). Reduced physical activity is frequently observed in patients with RA, which is another important cause of bone loss in RA (11).

Cytokines and growth factors are not the only factors modulating bone homeostasis. During daily activities bones are subjected to a variety of mechanical loads which affect bone remodeling and architecture. Osteocytes play a vital role in the adaptation of bone to mechanical loads since they translate mechanical stimuli into a biological response (12-14). Osteocytes sense mechanical stimuli and produce signaling molecules that are potent regulators of the recruitment and activity of bone-forming osteoblasts, as well as bone-resorbing osteoclasts and their precursors (10, 15-17). In response to mechanical stimuli osteocytes release bone anabolic factors such as NO, and PGE_2 (18, 19). Recently it has been reported that mechanically loaded osteocytes produce IL-6, which affects both osteoclasts and osteoblasts (20). Osteocytes release pro-osteoclastogenic signals in the absence of mechanical loading, leading to stimulation of bone resorption (21). In the presence of mechanical stimuli, osteocytes produce factors that inhibit osteoclastogenesis, and/or decrease the production of osteoclast-stimulating signals (22, 23). Mechanical stimuli affect the production of cytokines and signaling molecules such as IL-6, RANKL, OPG, CYR61, MEPE, COX-2, and sclerostin by osteocytes (23-25). IL-6 and RANKL enhance osteoclastogenesis, while OPG , MEPE, and CYR61 inhibit osteoclastogenesis (23-26). PGE2 enhances RANKL gene expression by osteoblasts (27). Sclerostin may have a catabolic action through promotion of osteoclast formation and activity by osteocytes, in a RANKL-dependent manner (28)."

Recombinant IL-1β and TNFα reduce the physiological response of osteocytes to mechanical loading (29, 30), but whether the inflammatory factors as present in the serum of patients with active RA affect the response of osteocytes to

mechanical stimuli is still unclear. Previously it has also been shown that mechanical loading of MLO-Y4 osteocytes is perfectly able to reduce the stimulatory effect of recombinant IL-1β on osteocyte to osteoclast communication (10). Whether mechanical loading is able to alter the stimulatory effect of RA-serum on human osteocyte-to-osteoclast signaling, resulting in changes in bone resorption, is unknown. In this study we tested the hypothesis that mechanical stimuli reverse the stimulatory effect of RA-serum on osteocyte-to-osteoclast communication.

METHODS

Recruitment of RA patients

RA patients with active stage of disease were recruited (mean age: 62±12 yrs; 6 females, 2 males), and diagnosed according to the 1987 RA classification at an early stage of the disease (less than 1 year disease duration) and before they had taken DMARDs or corticosteroids. Blood samples were collected, and within 1 h centrifuged for 10 min at 3000 rpm to separate the sera, that were aliquoted and stored at –80ºC. Patient characteristics, demographics, and clinical data (DAS score, Serum C-reactive Protein (CRP)) were collected (Table 1). Blood samples were also collected from age and gender-matched healthy controls. Patients with thyroid dysfunction, other inflammatory diseases besides RA, and pregnancy were excluded. The protocol was approved by the Ethical Review Board of the VU University Medical Center, Amsterdam, The Netherlands, and all subjects gave informed consent.

Table 1. Characteristics and demographics of the RA patients and healthy controls included in this study.

	Active RA patients	Healthy controls
Sex (female/male)	6/2	6/2
Age, yrs	62.3±12.1	62.1±12.4
DAS28 score	4.9±1.3	n.d.
CRP (mg/l)	36.2±40.3	<2.5
# RF-positive patients	2	n.d.

Values are mean±SD. Eight active RA patients were included in this study.
DAS28 score, disease activity score; CRP, C-reactive protein; RF, rheumatoid factor; n.d., not determined.

Human primary bone cell culture

Trabecular bone samples (surgical waste) from 2 male donors (age: 38 and 73 yrs) and 1 female donor (age: 55 yrs) were obtained from the anterior iliac crest during sinus floor elevation surgery using autologous anterior iliac crest bone graft. CRP levels of all donors was <2.5 mg/l, indicating no inflammatory disease in all donors. The protocol was approved by the Ethical Review Board of the VU University Medical Center, Amsterdam, The Netherlands, and all subjects gave informed consent.

Human primary bone cells were established as described earlier (30), and used as a model for osteocytes. Briefly, trabecular bone pieces were chopped into small fragments, and washed extensively with phosphate-buffered saline (PBS).

Bone fragments were then incubated with 2 mg/ml collagenase type II (Worthington, Freehold, NJ) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, UK)/Nutrient mixture F-12 (F-12) (DMEM/F-12, 1:1 (v/v)) for 2 h at 37ºC in a shaking water bath to remove all adhering cells from the bone chip surfaces. Bone fragments were then washed with medium containing 10% Fetal Clone I serum (HyClone, South Logan, UT), subdivided into equal portions, and transferred to 75 cm^2 culture flasks (Greiner Bio-One, Kremsmuenster, Austria). To obtain outgrowth of bone cells, bone fragments were cultured in DMEM/F-12 supplemented with 10% Fetal Clone I serum, 100 U/ml penicillin (Sigma, Hamburg, Germany), 100 µg/ml streptomycin sulfate (Gibco), 50 µg/ml gentamicin (Gibco), 1.25 µg/ml fungizone (Gibco), and 100 µg/ml ascorbate (Merck, Darmstadt, Germany) at 37ºC in a humidified atmosphere with 5% CO_2. The culture medium was refreshed twice a week. Cultures were continued till ~90% confluency.

Effect of active RA-serum and pulsating fluid flow (PFF) on osteocyte culture
Outgrowth bone cells between passages 1 to 3 were trypsinized with 0.25% trypsin (Difco Laboratories, Detroit, MI) and 0.1% EDTA (Sigma) in PBS. Cells were seeded at a density of $1x10^5$ cells/25 cm^2 culture flask (Nunc, Roskilde, Denmark) and incubated overnight. Then cells were cultured in DMEM with 10% RA-serum or healthy control-serum for 7 days to mimic chronic systemic inflammation during RA, and culture medium was refreshed after 3 days with medium also containing 10% RA-serum or healthy control serum. After 7 days, cells were trypsinized and seeded onto polylysine-coated (50 µg/ml poly-L-lysine hydrobromide, Sigma) glass slides (size 22x22 mm) at $5x10^4$ cells/glass slide, and cultured overnight in 6 well plates with DMEM containing 10% RA-serum or healthy control-serum. The next day culture medium was replaced by DMEM with 0.2% bovine serum albumin (BSA), and the cells were subjected to 1 h PFF as described previously (18, 19, 29, 31). Briefly, cells were subjected to PFF (mean±amplitude 0.7±0.7 Pa, 5 Hz) by pumping 4 ml of culture medium with 0.2% BSA through a parallel-plate flow chamber containing the osteocytes. Stationary control cultures were kept in 6 well plates under similar conditions as the experimental cultures, i.e. at 37ºC in a humidified atmosphere of 5% CO_2 in air. After 5 and 60 min of PFF or static culture, the medium was collected and assayed for NO and PGE_2 production. After 1 h PFF, treatment was terminated, and the cells were post-incubated in fresh DMEM with 0.2% BSA for 1 h without mechanical loading. After 1 h post-incubation the CM was collected and cells were processed for total RNA isolation.

NO and PGE_2 production
NO production was measured as nitrite (NO_2^-) accumulation in the CM using Griess reagent containing 1% sulfanylamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in non-CM were used

as a standard curve. Absorbance was measured at 540 nm with a microplate reader (Bio-Rad Laboratories). PGE_2 was measured by using PGE_2 High Sensitivity ELISA Kit (Abcam®, Cambridge, UK).

RNA isolation and real time RT-PCR

Total RNA from osteocytes was isolated using an RNeasy® Micro kit with an on-column DNase I digestion (Qiagen, Basel, Switzerland). Total RNA concentrations were measured with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA synthesis was performed in a thermocycler GeneAmp® PCR System 9700 PE (Applied Biosystems, Foster City, CA), using aSuperScript® VILO™ cDNA Synthesis Kit (LifeTechnologies, Inchinnan, UK), with 0.1 µg of total RNA in 20 µl reaction mixture consisting of VILO Reaction Mix and SuperScript Enzyme Mix. cDNA was stored at −20°C until real-time PCR analysis. Real-time PCR reactions were performed using 2.0 µl cDNA and SYBR® Green Supermix (Roche Laboratories, Indianapolis, IN) in a LightCycler® (Roche Diagnostics, Switzerland). In each PCR run, the reaction mixture without cDNA was used as a negative control. For quantitative real-time PCR, the values of relative target gene expression were normalized to relative housekeeping gene (YWHAZ) expression. Real-time PCR was used to assess expression of the following genes: COX-2, RANKL, OPG, MEPE, SOST and IL-6. All primers used for real-time PCR were from Life Technologies. The primer sequences are listed at Table 2. In each assay for osteogenic marker gene expression, mRNA preparations of human bone were used as a reference and internal control for the primer sets to pick up the specific mRNA of interest.

Osteoclastogenesis

Peripheral blood mononuclear cells (PBMCs) were isolated from a buffy coat (Sanquin, Amsterdam, The Netherlands) as described previously (32). PBMCs were seeded at $5x10^5$ cells/well of 96 well plates in DMEM containing 10% FCS, antibiotics (100 U/ml penicillin, 100 g/ml streptomycin, and 250 ng/ml amphotericin B), and control-serum–pretreated-CM (static and PFF), or RA-serum–pretreated-CM (static and PFF) (ratio DMEM:CM = 1:1 (v/v)). Twenty-five ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN) was added to the cells from day 1 to day 3. Ten ng/ml M-CSF and 4 ng/ml human RANKL (Peprotech, London, UK) were added from day 3 to day 21. After 3 weeks of culture, cells were fixed in 4% formaldehyde, and stained for tartrate-resistant acid phosphatase (TRACP; TRAP Kit, Sigma). Nuclei were visualized by 4',6-diamidino-2-phenylindole (DAPI) staining. Osteoclast formation was assessed by counting the number of TRACP-positive multinucleated cells (MNCs), containing 3 or more nuclei per cell. Osteoclasts were counted in 5 fixed microscopic fields of each well using a Leica DM IL microscope (Leica, Wetzlar, Germany) equipped with a 20x objective.

Statistical analysis

Data are expressed as mean. The effects of RA-serum or PFF on NO and PGE_2 production, gene expression of cytokines and growth factors, and osteoblast-mediated osteoclastogenesis were tested by one-way analysis of variance (ANOVA). ANOVA was applied to the four groups for each parameter analyzed, followed by Bonferroni's multiple comparison test as post hoc test. Differences were considered significant if $p<0.05$. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA).

Table 2. Primers used in the real-time PCR assay.

Gene		Oligonucleotide Sequence	Amplicon length (bp)
YWHAZ	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'	
COX2	Forward	5' GCATTCTTTGCCCAGCACTT 3'	299
	Reverse	5' AGACCAGGCACCAGACCAAAGA 3'	
CYR61	Forward	5' CAACCCTTTACAAGGCCAGA 3'	206
	Reverse	5' TGGTCTTGCTGCATTTCTTG 3'	
IL-6	Forward	5' ACAGCCACTCACCTCTTCA 3'	207
	Reverse	5' ACCAGGCAAGTCTCCTCAT 3'	
OPG	Forward	5' TGGAATAGATGTTACCCTGTGTG 3'	298
	Reverse	5' GCTGCTCGAAGGTGAGGTTA 3'	
RANKL	Forward	5' CATCCCATCTGGTTCCCATAA 3'	60
	Reverse	5' GCCCAACCCCGATCATG 3'	
SOST	Forward	5' GGGTGGCAGGCGTTCA 3'	164
	Reverse	5' CTGTACTCGGACACGTCTTTGGT 3'	
MEPE	Forward	5' GAGTTTTCTGTGTGGGACTACTCCTT 3'	101
	Reverse	5' TCTGCTCTTCCACACAGCTTTG 3'	

RESULTS

RA-serum enhanced osteoclastogenic gene expression by osteocytes, while PFF attenuated this effect

We analyzed the effect of RA-serum as well as the combination of RA-serum and PFF on osteoclastic gene expression by primary osteocytes. Cells were subjected to PFF after 7 days of culture with RA-serum or control-serum. RA-serum enhanced RANKL gene expression by 2-fold in static osteocytes (Fig. 1A). It inhibited OPG gene expression by 2.7-fold in static osteocytes and 2.8-fold in PFF-subjected osteocytes (Fig. 1B). RA-serum enhanced the RANKL/OPG ratio by 3.4-fold in static osteocytes, while PFF treatment of RA-serum–pretreated osteocytes nullified this effect (Fig. 1C). PFF enhanced CYR61 gene expression by 2.7-fold in control-serum–pretreated osteocytes, and 2.3-fold in RA-serum–pretreated osteocytes (Fig. 1D). PFF upregulated IL-6 gene expression in control osteocytes by 28-fold, and in RA-serum–pretreated osteocytes by 33-fold (Fig. 1E). PFF upregulated COX2 gene expression in control osteocytes by 6.4-fold, and in RA-serum–pretreated osteocytes by 10-fold (Fig. 1F). SOST and MEPE gene expression were not affected by RA-serum nor by PFF treatment (Fig. 1G and Fig. 1H).

PFF enhanced NO but not PGE_2 production in the presence of RA-serum

PFF enhanced NO production by 2.6-fold at 5 min (Fig. 2A), and by 3.5-fold at 60 min (Fig. 2B) in control serum treated osteocytes. Similarly, PFF treatment of RA-serum–pretreated osteocytes enhanced NO production by 2.7-fold at 5 min (Fig. 2A), and by 3.6-fold at 60 min (Fig. 2B). RA-serum pretreatment alone did not affect NO production. RA-serum pretreatment and the combination of RA-serum and PFF treatment did not affect PGE_2 production by osteocytes at 5 min (Fig. 2C). PFF enhanced PGE_2 production by 2.5-fold in control-serum–pretreated osteocytes at 60 min, but not in RA-serum–pretreated osteocytes (Fig. 2D).

RA-serum–pretreated osteocytes enhanced osteoclast formation, while PFF nullified this effect

PFF-CM from control-serum–pretreated osteocytes decreased the number of TRACP-positive osteoclasts with 3-5 nuclei by 1.6-fold (Fig. 3, A and B). Stat-CM from RA-serum–pretreated cell culture increased the number of TRACP-positive osteoclasts with 3-5 nuclei by 1.2-fold, while PFF nullified this effect (Fig. 3, A and B). PFF-CM from RA-serum–pretreated osteocytes also inhibited formation of osteoclasts with >5 nuclei by 1.8-fold (Fig. 3, A and C).

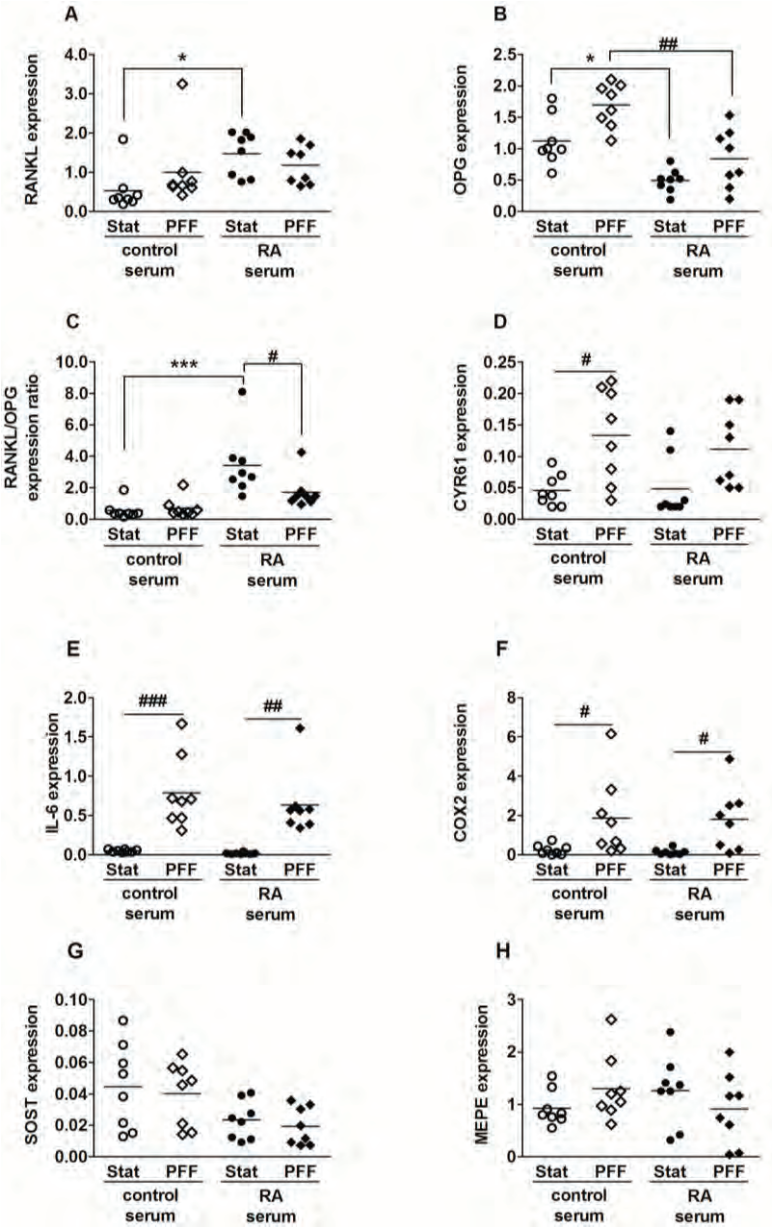

Figure 1. Effect of RA-serum and/or PFF treatment on cytokine and growth factor/inhibitor gene expression by primary osteocytes. Cells were cultured for 7 days with or without RA-serum, followed by 1 h PFF or static control culture, and 1 h post-incubation without PFF. (A) RA-serum enhanced RANKL gene expression. (B) RA-serum inhibited OPG gene expression. It also reduced the stimulatory effect of PFF on OPG gene expression. (C) RA-serum enhanced RANKL/OPG gene expression ratio, and PFF nullified this effect. (D) PFF

enhanced CYR61 gene expression in control and RA-serum–pretreated osteocytes. (E) PFF enhanced IL-6 gene expression in control and RA-serum–pretreated osteocytes. (F) PFF enhanced COX2 gene expression in control and RA-serum–pretreated osteocytes. (G) RA-serum and/or PFF did not affect SOST gene expression, nor (H) MEPE gene expression. Values are mean from 8 independent experiments. Significant effect of RA-serum, *p<0.05, ***p<0.001. Significant effect of PFF, #p<0.05, ##p<0.01, ###p<0.001. Stat, static control culture; PFF, pulsating fluid flow.

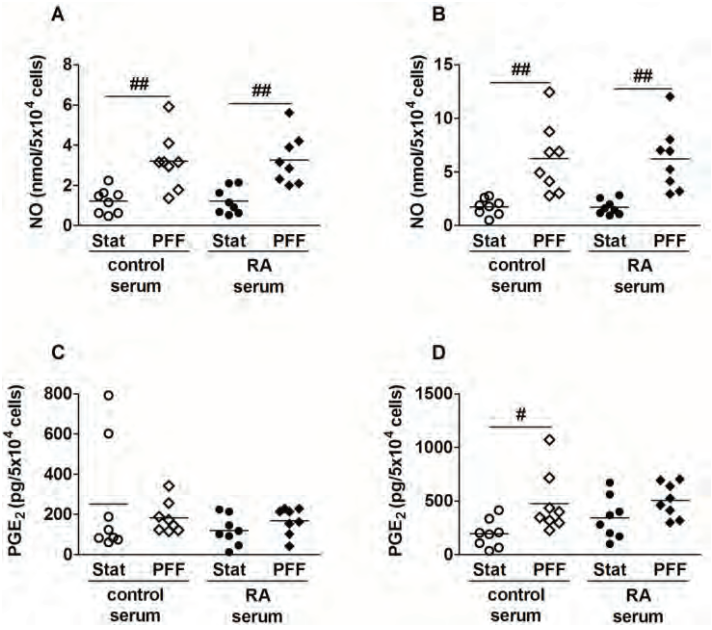

Figure 2. Effect of PFF on NO and PGE_2 production by control-serum and RA-serum–pretreated primary osteocytes. (A) PFF enhanced NO production by control-serum and RA-serum–pretreated osteocytes at 5 min, and (B) at 60 min. (C) PFF did not affect PGE_2 production in control-serum or RA-serum–pretreated osteocytes at 5 min. (D) PFF enhanced PGE_2 production in control-serum, but not RA-serum–pretreated osteocytes at 60 min. Values are mean from 8 independent experiments. Significant effect of PFF, #p<0.05, ##p<0.01.

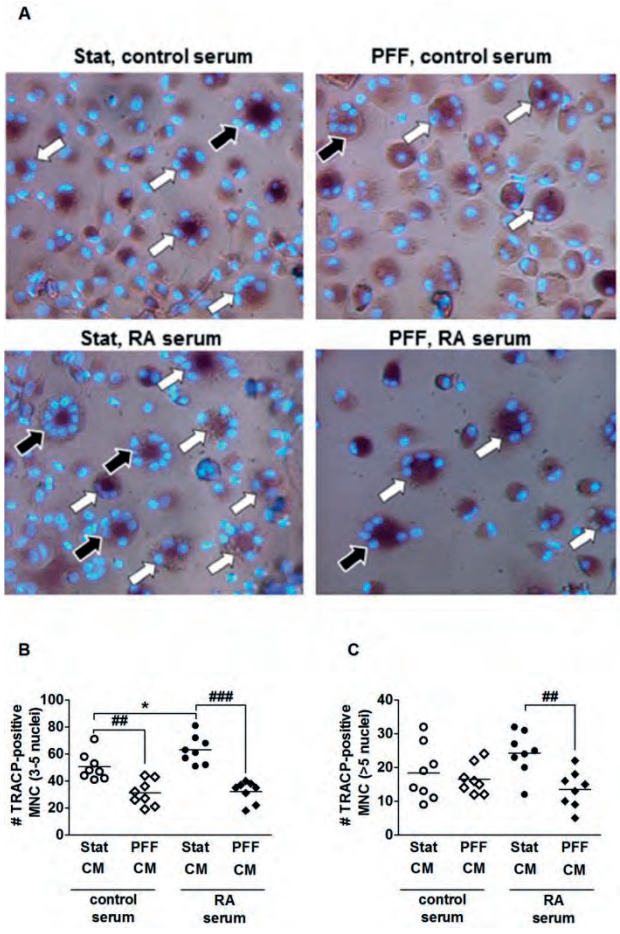

Figure 3. Effect of Stat-CM from RA-serum–pretreated osteocytes and PFF-CM from RA-serum–pretreated osteocytes on osteoclast formation. CM was obtained from osteocytes cultured for 7 days with or without RA-serum, followed by 1 h PFF or static control culture, and 1 h post-incubation without PFF. CM was added to osteoclast precursors. (A) Representative micrograph of TRACP-positive multinucleated cells (TRAP+ MNC) in a culture of human PBMCs with stat-CM from control-serum–pretreated osteocytes, PFF-CM from control-serum–pretreated osteocytes, stat-CM from RA-serum–pretreated osteocytes, and PFF-CM from RA-serum–pretreated osteocytes. White arrows: TRACP+ MNC with 3-5 nuclei; black arrows: TRACP+ MNC with >5 nuclei. (B) PFF-CM from control-serum–pretreated osteocytes inhibited the formation of osteoclasts with 3-5 nuclei. Stat-CM from RA-serum–pretreated osteocytes enhanced the formation of osteoclasts with 3-5 nuclei, and PFF-CM from RA-serum–pretreated osteocytes nullified this effect. (C) PFF-CM from RA-serum–pretreated osteocytes inhibited the formation of osteoclasts with >5 nuclei. Values are mean from 3 independent experiments, n=9. Significant effect of Stat-CM from RA-serum–pretreated osteocytes, *$p<0.05$. Significant effect of PFF-CM, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.

DISCUSSION

Systemic inflammation and immobilization are associated with bone loss in RA (11). In this study we tested the hypothesis that mechanical stimuli reverse the stimulatory effect of RA-serum on osteocyte-to-osteoclast communication. We found that RA-serum did not affect the *intrinsic* capacity of osteocytes to sense mechanical stimuli, i.e. the osteocytes showed an unchanged NO response to PFF, although PGE_2 production was affected, and an unaltered gene expression of IL-6, COX2, CYR61, MEPE, and SOST. RA-serum upregulated osteocyte-to-osteoclast communication and osteocyte-mediated osteoclastogenesis. Interestingly, pulsating fluid flow attenuated the stimulatory effect of RA-serum on osteocyte-to-osteoclast communication and osteocyte-mediated osteoclastogenesis. Our data indicate that mechanical stimuli on osteocytes may prevent inflammation driven osteocyte-mediated osteoclastogenesis in RA.

We found that RA-serum enhanced the gene expression ratio of RANKL/OPG in primary osteocytes. Moreover, CM from RA-serum–pretreated osteocytes enhanced osteoclast formation. These findings are in accordance with our previous findings showing that RA-serum–pretreated human primary osteoblasts enhance osteoclastogenesis (8). Kulkarni and colleagues showed that IL-1β not only enhances the gene expression ratio of RANKL/OPG in osteocytes, but it also stimulates osteocyte-mediated osteoclastogenesis in a dose-dependent manner (10). In this study we found that RA-serum did not affect IL-6, CYR61, COX2, SOST, or MEPE gene expression, even though we previously found that RA-serum enhances IL-6 gene expression in primary human osteoblasts (8). This contradiction between current findings and earlier observations might be time-point related, i.e. in this study we analyzed IL-6 gene expression after 7 days of RA-serum treatment, while in our previous study IL-6 expression was assessed after 10 days of treatment with RA-serum from a different RA patient group. Our data indicate that RA-serum containing inflammatory cytokines enhances osteocyte-to-osteoclast communication, which may contribute to generalized osteoporosis in RA.

Inflammatory cytokines such as IL-1β and TNFα have been reported to reduce the mechanosensitivity of mouse osteocytes (29). We found that RA-serum did not affect the mechanosensitivity of human osteocytes, which might be explained by the multitude of factors present in RA-serum, such as growth factors and their antagonists, pro-inflammatory as well as anti-inflammatory cytokines, cytokine receptors, and antibodies to cytokines. The combined effect of all these factors on the response of osteocytes to mechanical loading might be different from the effect of an individual recombinant cytokine. PFF nullified the stimulatory effect of RA-serum on the RANKL/OPG expression ratio in osteocytes. Similarly PFF treatment of RA-serum–pretreated osteocytes nullified the stimulatory effect of RA-

serum on bone cell-mediated osteoclastogenesis. Our findings are in accordance with data obtained by Kulkarni and colleagues showing that IL-1β enhances osteocyte-mediated osteoclastogenesis, and that mechanical loading reduces this effect (10). We found that PFF enhanced IL-6, COX2, and CYR61 gene expression to a similar extent in RA-serum and control-serum treated osteocyte cultures, but it did not affect SOST and MEPE gene expression. Kulkarni and colleagues showed that PFF enhances MEPE expression in a murine osteocyte cell line (MLO-Y4) (23), while Robling and colleagues reported that high strain mechanical loading reduces sclerostin levels in mouse ulnae *in vivo* (25, 33). Mechanical loading applied via oscillatory fluid flow for 2 h, but not 1 h, reduces SOST expression in UMR 106.01 osteoblasts (34). This discrepancy might be due to differences in the cell types used, the magnitude and the type of loading applied, and the experimental set up such as *in vitro* 2D culture and an *in vivo* mouse model. We applied a peak shear stress of only 0.7 Pa for 1 h, while Papanicolaou and colleagues applied a peak shear stress of 2.0 Pa for 2 h on UMR 106.01 osteoblasts resulting in reduced SOST gene expression (34). Moreover we applied pulsating fluid flow on osteocytes for only 1 h, while Robling and colleagues loaded mice ulna *in vivo* by 360 cycles/day for two consecutive days (25). In our study the duration of mechanical loading and/or applied peak shear stress might not have been enough to affect SOST gene expression. We found that PFF did not inhibit the RANKL/OPG gene expression ratio in osteocytes treated with control serum. The RANKL/OPG pathway is important for osteoclastogenesis, but many other osteoclastogenesis-modulating signaling molecules are produced by osteocytes as well (23, 26). In this study we observed enhanced gene expression of CYR61 by osteocytes treated with control serum, which might explain the decreased osteoclastogenesis in cultures of osteocyte precursors with PFF-CM from control-serum-pre-treated osteocytes, since CYR61 inhibits osteoclastogenesis (26). We found that PFF reduced both primary osteocyte-mediated osteoclastogenesis and the stimulatory effect of RA-serum on primary osteocyte-mediated osteoclastogenesis. The effect of rheumatoid factor-positive and negative sera on cytokine and growth factor gene expression by osteocytes and on osteocyte-mediated osteoclastogenesis was similar. Our findings suggest an important role of mechanical loading in the inhibition of both physiological as well as inflammation-induced osteoclastogenesis. Based on our findings, we created a pathophysiological model illustrating how mechanical loading might prevent inflammation induced bone loss in RA (Fig. 4).

A strength of our study is that we used a well-defined RA patient group as well as a well-defined healthy control group. We also used a well established mechanical loading system applying pulsating fluid flow resulting in fluid shear stress on a monolayer of cells. In addition we used well-established cell types, such as human primary osteocytes and human PBMCs. Our human primary

osteocyte model is using primary bone cells, which have been shown to exhibit both an osteocyte and osteoblast-like phenotype (18, 31, 35, 36). These cells are more close to the *in vivo* human bone niche in comparison to human or mouse osteoblast cell lines or primary mouse or chicken osteocytes. The relatively high expression of the osteocyte-specific genes MEPE and SOST compared to the housekeeping gene indicates the osteocytic nature of the cells used in this study. Today there is no better option than using human primary bone cells as a model for human osteocytes. Here osteoclast precursors were cultured with a mixture of CM (containing 0.2% BSA) obtained from osteocytes pretreated with RA-serum or control serum, and fresh DMEM (containing 10% fetal clone serum) (CM:DMEM, 1:1, v/v). Healthy control serum and RA-serum contain antibodies and autoantibodies that reduce osteoclastogenesis by inhibiting cytokine and signaling molecule function (37, 38). In this study osteoclast precursors were never in contact with RA-serum or healthy control serum, thereby eliminating a possible direct effect of antibodies and autoantibodies present in the RA-serum or healthy control serum on osteoclastogenesis. A limitation of our study might be the relatively low number of patients included. Statistical significance between groups is not easily obtained due to the fairly large data variation, probably due to donor variation. Another limitation of our study might be possible differences in the potency of the serum as a result of differences in storage time of the serum samples. The potency of serum decreases with storage time, and therefore active RA-serum stored for more than one year might have lost some of its potency more so than serum stored for a shorter time.

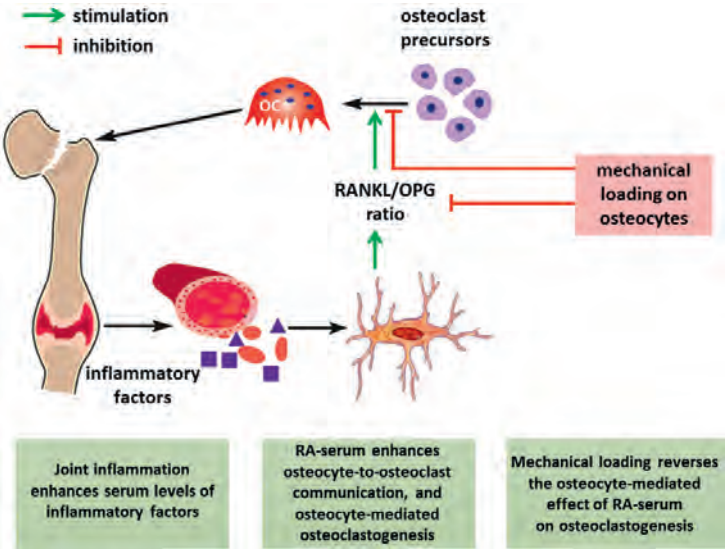

Figure 4. Pathophysiological model illustrating how mechanical loading can prevent inflammation-induced bone loss in RA. Whether mechanical loading-stimulated osteocytes produced factors that inhibit osteoclast activity was not tested in this study.

In conclusion, we found that RA-serum did not alter the intrinsic capacity of osteocytes to sense mechanical stimuli, but upregulated osteocyte-to-osteoclast signaling, while mechanical loading nullified this effect. Our data suggests that mechanical loading of bone might prevent osteoclast-related bone loss in RA patients by reversing the stimulatory effect of RA-serum on osteocyte-to-osteoclast signaling. Physical activity or other forms of bone loading, e.g. vibrating platforms, could thus have great therapeutic potential in the prevention of osteoporosis in RA and other inflammatory diseases.

ACKNOWLEDGEMENTS

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CHAPTER 7

Inflammatory Cytokines Affect Gene Expression of Osteocyte Signaling Molecules by Human Bone Cells Cultured in Their Native Matrix

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Manuscript in preparation

ABSTRACT

Bone remodeling is disturbed in rheumatoid arthritis (RA), leading to bone loss, possibly as a result of elevated levels of circulating inflammatory cytokines. Osteocyte signaling plays a vital role in bone mass regulation by orchestrating bone formation and/or bone resorption, but the effect of inflammatory cytokines on osteocyte signaling remains to be elucidated. Therefore we aimed to investigate the effect of RA-serum or exogenous recombinant inflammatory cytokines and chemokines on human osteocyte signaling molecule production.

Human trabecular bone chips were denuded by collagenase-2 treatment for 2 h. Then bone chips, containing osteocytes embedded in their native matrix, were cultured ± 10% active RA-serum, or ± 10 ng/ml recombinant IL-1β, IL-6, IL-17, or TNFα, 200 pg/ml IL-8, 500 pg/ml CCL20, or a combination of IL-1β, TNFα, and IL-6 for 7 days. Live-dead staining was performed to assess cell viability. Gene expression of cytokines and osteocyte signaling proteins was analyzed by qPCR. Immuno-staining was performed for sclerostin.

Approximately 60% of the osteocytes in the bone chips were alive at day 7. Osteocytes in bone chips did express mRNA for the osteocyte markers sclerostin, FGF23, DMP1, and MEPE, and the cytokines IL-1β, IL-6, and TNFα at day 0 and 7. Osteocytes in bone chips were positive for sclerostin immuuno-staining. RA-serum, IL-1β, TNFα, and the combination of IL-1β, TNFα, and IL-6 enhanced IL-1β gene expression (3.3 to 80–fold). RA-serum, IL-8, CCL20, IL-1β, IL-6, TNFα, and the combination of IL-1β, TNFα, and IL-6 enhanced TNFα gene expression (2 to 3.5–fold). CCL20, IL-17, IL-1β, TNFα, and the combination of IL-1β, TNFα, and IL-6 enhanced IL-6 expression (3 to 87–fold). IL-8, CCL20, IL-1β, TNFα, and the combination of IL-1β, TNFα, and IL-6 enhanced IL-8 gene expression (5 to 233–fold). IL-8, CCL20, IL-1β, TNFα, and the combination of IL-1β, TNFα, and IL-6 enhanced FGF23 gene expression (2.3 to 3.9–fold).RA-serum, IL-1β, and the combination of IL-1β, TNFα, and IL-6 enhanced SOST gene expression (2.5 to 8.5–fold). RA-serum enhanced DKK1 gene expression (2–fold).

Active RA-serum, individual exogenous recombinant cytokines, chemokines, and a combination of cytokines modulated gene expression of cytokines, phosphate homeostasis-related signaling molecules, and Wnt inhibitors in human osteocytes cultured in their native matrix. These results suggests that osteocytes could be a new target in the prevention of bone loss in inflammatory diseases.

KEY WORDS:

Rheumatoid arthritis, inflammation, cytokine, chemokine, osteocyte signaling, bone loss

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by local bone erosion, joint space narrowing, and extra-articular manifestation such as generalized osteoporosis (1, 2). Generalized osteoporosis in RA is in part caused by immobility and corticosteroid therapy (3, 4), but it has also been attributed to the effects of chronic systemic inflammation, such as elevated levels of circulating cytokines. Cells in inflamed synovia in RA produce high amounts of growth factors, as well as cytokines and chemokines such as interleukin-1β (IL-1β), IL-6, IL-17, tumor necrosis factor-α (TNFα), CXCL8 (IL-8), CXCL9, CXCL10, and CCL20 (5, 6). Elevated levels of these cytokines and chemokines are found in synovial fluid and serum from RA patients (6-10). Since bone is a well vascularized tissue, elevated levels of inflammatory cytokines in the circulation during systemic inflammation can easily reach the bone cells.

Inflammatory cytokines affect osteoblast and osteoclast formation and activity, as well as the physiological response of osteocytes to mechanical stimuli, thereby disturbing the delicate balance between bone formation and resorption during bone remodeling (5, 11, 12). Osteocytes reside within the bone matrix, and comprise about 90 to 95% of the cellular component in bone tissue. The primary function of osteocytes is to sense mechanical stimuli and deliver signals to osteoblasts and osteoclasts (13, 14). Activation of canonical Wnt signaling exclusively in osteocytes induces bone anabolism and triggers Notch signaling (13). Moreover osteocytes also have a function in phosphate homeostasis (14). For this purpose osteocytes produce a range of cytokines and signaling molecules including dentin matrix protein 1 (DMP1), phosphate-regulating neutral endopeptidase on chromosome X (PHEX), Dickkopf-related protein 1 (DKK1), matrix extracellular phosphoglycoprotein (MEPE), fibroblast growth factor 23 (FGF23), sclerostin (SOST), osteoprotegerin (OPG), and receptor activator of nuclear factor-κB ligand (RANKL) (15-19). RANKL and OPG regulate osteoclast formation and activity (20, 21). Sclerostin and DKK1 inhibit bone formation by inhibiting the stimulatory effect of Wnts on osteoblast differentiation and activity (16, 22). DMP1 regulates bone mineralization, while FGF23, MEPE, and PHEX are associated with phosphate homeostasis (23), as well as regulate production of 1,25 dihydroxyvitamin D_3 (23-25). Circulating proinflammatory cytokines may affect the expression of these signaling molecules by osteocytes. Recombinant IL-1β, IL-6, and TNFα enhance osteocyte-mediated osteoclastogenesis (18, 26). Moreover IL-1β and TNFα enhance RANKL, IL-6, TNFα, and FGF23 production by osteocytes (17, 18, 26). Systemic inflammation might thus directly affect osteocyte signaling, thereby disturbing bone remodeling and phosphate homeostasis in patients. Yet, the effect of individual exogenous recombinant cytokines or a complex mixture of

cytokines and signaling molecules present in the circulation of patients with chronic inflammation on osteocyte signaling is so far unknown.

Cultured human bone chips containing osteocytes embedded in their matrix can be used to study the effect of systemic inflammation on osteocyte signaling (19). In this study we aimed to investigate the effect of exogenous recombinant cytokines, chemokines, a combination of cytokines, and serum of patients with active RA on gene expression of a panel of cytokines and signaling molecules by osteocytes cultured in their native extracellular matrix.

MATERIALS AND METHODS

Recruitment of RA patients

RA patients with an active stage of disease (mean age: 62±12 yrs; 6 females, 2 males), and diagnosed according to the 1987 RA classification at an early stage of the disease (less than 1 year disease duration) were recruited before they had taken DMARDs or corticosteroids. Blood samples were collected, and within 1 h centrifuged for 10 min at 3000 rpm to separate the sera, that were aliquoted and stored at –80ºC. Patient characteristics, and clinical data (DAS score, Serum C-reactive Protein (CRP)) were collected (Table 1). Blood samples were also collected from age and gender-matched healthy controls. In addition, a healthy control sera pool was made by pooling the sera from 22 healthy donors (age: 40.5 ± 17.7 yrs; 14 females, 8 males). The serum CRP level of the individual healthy control sera, as well as the healthy control pool was <2.5 mg/l, which indicates that all healthy donors did not suffer from inflammatory disease during the time of serum collection. Patients with thyroid dysfunction, other inflammatory diseases besides RA, and pregnancy were excluded. The protocol was approved by the Ethical Review Board of the VU University Medical Center, Amsterdam, The Netherlands, and all subjects gave informed consent.

Table 1. Characteristics and clinical data of the RA patients included in this study.

	active RA patients
Sex (female/male)	9/4
Age, yrs	56.2±14.3
DAS28 score	5.1±1.2
CRP (mg/l)	23.9±34.8

Values are mean±SD. Eight active RA patients were included in this study.
DAS28 score, disease activity score; CRP, C-reactive protein.

Human bone cells cultured in close contact with their native matrix

Trabecular bone samples (surgical waste) from 5 female and 3 male donors (age: 42±16 yrs) were obtained from the anterior iliac crest during sinus floor elevation surgery using autologous anterior iliac crest bone graft. The serum CRP level of the donors was <2.5 mg/l, which indicates that all healthy donors did not suffer from inflammatory disease during the time of serum collection. The protocol was approved by the Ethical Review Board of the VU University Medical Center, Amsterdam, The Netherlands, and all subjects gave informed consent.

Trabecular bone was chips were prepared and denuded as described previously (27). Briefly, trabecular bone fragments were placed in sterile phosphate-buffered saline (PBS), chopped into small fragments, and washed extensively with PBS. Bone fragments were then incubated with 2 mg/ml collagenase type II (Worthington, Freehold, NJ) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY)/Nutrient mixture F12 (F12) (DMEM/F12, 1:1 (vol/vol)) for 2 h at 37°C in a shaking waterbath to remove all adhering cells from the bone chip surfaces. Denuded bone chips were then washed extensively with PBS, followed by addition of medium containing 10% human pooled control serum, and transferred at 150 mg bone tissue/well of 12 well culture plates (Greiner Bio-One, Frickenhausen, Germany). Bone chips were cultured overnight in DMEM/F-12 supplemented with 2% platelet lysate, 10 U/ml heparin (Leo Pharma, Amsterdam, The Netherlands), 100 U/ml penicillin (Sigma, Hamburg, Germany), 100 µg/ml streptomycin sulfate (Gibco), 50 µg/ml gentamicin (Gibco), and 1.25 µg/ml fungizone (Gibco) at 37°C in a humidified atmosphere with 5% CO_2. The following day, the bone chips were washed 6 times with PBS, and cultured with medium containing 10% human serum. At this time point the trabecular bone chips look like clear sponge pieces, only consisting of bone cells embedded in their matrix.

Exogenous recombinant cytokine, chemokine, and active RA-serum treatment

Denuded bone chips were cultured with DMEM/F12 containing 10% active RA-serum or 10% human serum from age and gender-matched healthy control subjects, or 10% control pooled human serum. Exogenous recombinant human IL-1β (10 ng/ml; Sigma), IL-6 (10 ng/ml; R&D Systems, Minneapolis, MN), IL-17 (10 ng/ml; R&D Systems), TNFα (10 ng/ml; Sigma), IL-8 (200 pg/ml; Sigma), CCL20 (500 pg/ml; Sigma), or a combination of IL-1β, TNFα, and IL-6 (10 ng/ml each) was added to the bone chips cultures with 10% control pooled human serum. Treatment with cytokines, chemokines, a combination of cytokines, or active RA-serum was performed for 7 days. Medium with additives were replenished twice a week. The viability of cells in or on the bone chips was analyzed by ethidium homodimer-1 staining (Life technologies, Carlsbad, CA) at day 7. Live cells in or on the bone chips were observed with a Zeiss Apotome.2 microscope (Carl Zeiss, Oberkochen, Germany). At day 7, the cultures were terminated and the conditioned medium collected, and the bone chips put into TRIzol reagent (Life Technologies). After 7 days, some bone chips were fixed in cold 4% phosphate-buffered formaldehyde, dehydrated in graded ethanol, and embedded in methylmethacrylate (MMA; BDH Chemicals, Poole, England) supplemented with 20% plastoid-N (Röhm und Haas, Darmstadt, Germany), 2.0 g/L benzoylperoxide (Merck, Darmstadt, Germany), and

N,N-dimethylaniline (Merck) (28). Sections of 5 µm were cut with a Leica/Reichert-Jung Polycut S (SM2500) microtome (Nussolch, Germany).

Immunohistochemistry

Sections were rehydrated and endogenous peroxidase was quenched with 3% H_2O_2 in 40% methanol in PBS. Antigen retrieval was performed by incubation with 1% trypsin for 15 min at 37°C. After blocking of the non-specific binding sites with 5% normal rabbit serum for 1 h, the sections were incubated overnight at 4°C with 1/200 mouse-anti-SOST antibody (R&D Systems). The sections were incubated for 1 h with 1/100 biotinylated rabbit-anti-mouse antibody (Dako), and for 1 h with horse radish peroxidase-labeled streptavidin (Invitrogen). For color development the sections were incubated with 3-amino-9-ethylcarbazole (AEC) reagent (Life Technologies) and counterstained with hematoxylin. Sections of bone biopsies from patients with Crohn's disease were used as a positive control. Specific staining for SOST was visualized using light microscopy.

RNA isolation and real-time PCR

Total RNA was isolated from the cells in or on the bone chips using TRIzol reagent. Total RNA was further purified by using RNeasy® Micro kit with an on-column DNase I digestion (Qiagen, Basel, Switzerland). Total RNA concentrations were determined with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). cDNA synthesis was performed in a thermocycler GeneAmp® PCR System 9700 PE (Applied Biosystems, Foster City, CA), using a First Strand cDNA Synthesis Kit (Thermo Scientific), with 0.1 µg total RNA in 20 µl reaction mixture. cDNA was stored at −20°C until real-time PCR analysis. Real-time PCR reactions were performed using 2.0 µl cDNA and SYBR® Green Supermix (Roche Laboratories, Indianapolis, IN) in a LightCycler® (Roche Diagnostics, Basel, Switzerland). In each PCR run, the reaction mixture without cDNA was used as a negative control. For quantitative real-time PCR, the values of relative target gene expression were normalized relative to housekeeping gene (YWHAZ) expression. Real-time PCR was used to assess the expression of the following genes: RANKL, OPG, IL-1β, IL-6, IL-8, TNFα, MEPE, sclerostin gene (SOST), FGF23, and DKK1. All primers used for real-time PCR were from Life Technologies. The primer sequences are listed at Table 2.

Statistical analysis

Data are expressed as mean±SEM. The effects of cytokines, chemokines, a combination of cytokines, and RA-serum on gene expression of cytokines and growth factors were tested by one-way variance of analysis (ANOVA) followed by Dunnett's multiple comparison test as post hoc test. Differences were considered

significant if $p<0.05$. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, CA).

Table 2. Primers used in the real-time PCR assay

Gene		Oligonucleotide Sequence	Amplicon length (bp)
YWHAZ	Forward	5' GATGAAGCCATTGCTGAACTTG 3'	229
	Reverse	5' CTATTTGTGGGACAGCATGGA 3'	
DKK1	Forward	5' GCATGCGTCACGCTATGT 3'	271
	Reverse	5' TTACAGATCTTGGACCAGAA 3'	
DMP1	Forward	5' TAGGCTAGCTGGTGGCTTCT 3'	375
	Reverse	5' AACTCGGAGCCGTCTCCAT 3'	
IL-1β	Forward	5' TGGAGCAACAAGTGGTGTTCT 3'	270
	Reverse	5' GAGAGGTGCTGATGTACCAGTT 3'	
IL-6	Forward	5' ACAGCCACTCACCTCTTCA 3'	207
	Reverse	5' ACCAGGCAAGTCTCCTCAT 3'	
IL-8	Forward	5' TCTGCAGCTCTGTGTGAAG 3'	147
	Reverse	5' TGTGTTGGCGCAGTGTGG 3'	
FGF23	Forward	5' TGAGCGTCCTCAGAGCCTAT 3'	115
	Reverse	5' TTGTGGATCTGCAGGTGGTA 3'	
MEPE	Forward	5' GAGTTTTCTGTGTGGGACTACTCCTT 3'	101
	Reverse	5' TCTGCTCTTCCACACAGCTTTG 3'	
OPG	Forward	5' TGGAATAGATGTTACCCTGTGTG 3'	298
	Reverse	5' GCTGCTCGAAGGTGAGGTTA 3'	
RANKL	Forward	5' CATCCCATCTGGTTCCCATAA 3'	60
	Reverse	5' GCCCAACCCCGATCATG 3'	
SOST	Forward	5' GGGTGGCAGGCGTTCA 3'	164
	Reverse	5' CTGTACTCGGACACGTCTTTGGT 3'	
TNFα	Forward	5' AGAGGGCCTGTACCTCATCT 3'	315
	Reverse	5' AGGGCAATGATCCCAAAGTAG 3'	

RESULTS

Live osteocytes expressing osteocyte-specific markers as visualized in denuded bone chips

Around 60% of the osteocytes in the denuded bone chips were alive at day 7 (Fig. 1A). The bone cells cultured in their native matrix did express sclerostin protein at day 7 (Fig. 1B). Some live cells had crawled out and attached on the surface of the denuded bone chips at day 7 (data not shown). Bone cells cultured in their native matrix did express mRNA of osteocyte-specific signaling molecules, which are only hardly expressed by osteocytes cultured in 2D. The bone cells did express clearly detectable levels of SOST and DKK1 at day 0, while SOST expression was enhanced by 4-fold and DKK1 expression by 90-fold at day 7 compared to day 0 (Fig 2A). The bone cells also expressed MEPE and FGF23 at day 0, but MEPE expression was decreased by 16-fold and FGF23 expression by 14-fold between day 0 and 7 (Fig 2B). Hereafter we refer to these bone cells as “osteocytes”. Osteocytes did express RANKL and OPG mRNA at day 0 and 7; RANKL expression was 9-fold higher and OPG expression was 8-fold higher at day 7 compared to day 0 (Fig 2C). Osteocytes also expressed IL-1β, TNFα, IL-6, and IL-8 at day 0 and 7 (data not shown).

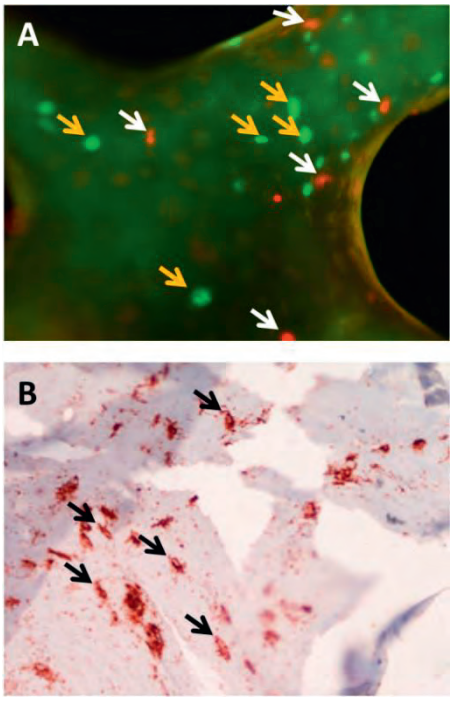

Figure 1. Bone cells express sclerostin protein in denuded bone chips after 7 days of culture. (A) Live and dead bone cells in denuded bone chips visualized by live-dead staining (yellow arrows, live cells; white arrows, dead cells). (B) Bone cells cultured in their native matrix highly express sclerostin protein (black arrows).

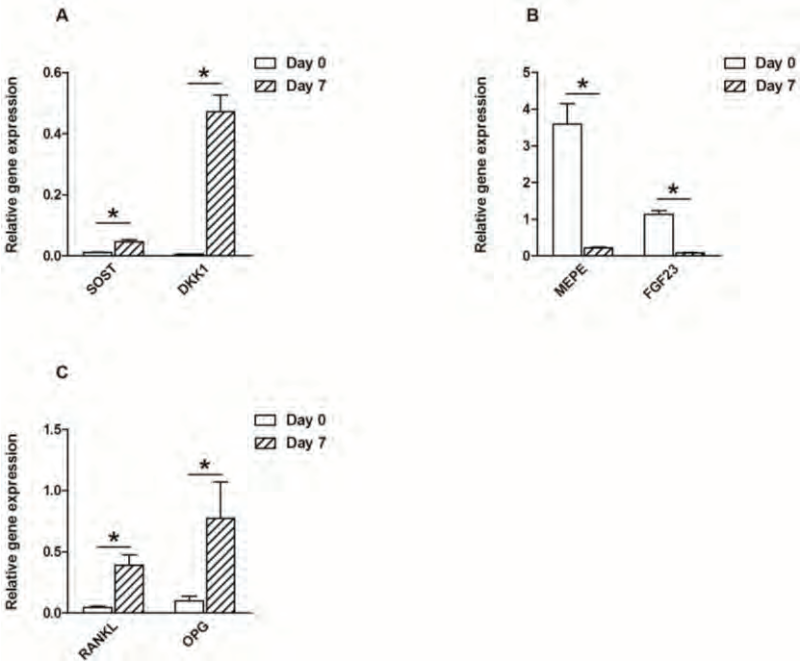

Figure 2. Bone cells cultured in their native matrix change osteocyte-specific gene expression after 7 days of culture. (A) Sclerostin and DKK1 gene expression was enhanced at day 7. (B) MEPE and FGF23 gene expression was reduced at day 7. (C) RANKL and OPG gene expression was enhanced at day 7. Values are mean±SEM from 3 experiments. The bone biopsy was obtained from 1 donor. Significantly different from day 0, *p<0.05.

RA-serum, exogenous cytokines, and chemokines enhance cytokine gene expression by osteocytes

Osteocytes cultured in their native matrix did express IL-1β, TNFα, IL-6, and IL-8 mRNA at day 7. The expression of these genes was affected by RA-serum, and by treatment with exogenous cytokines, chemokines, and a combination of cytokines. IL-1β gene expression was enhanced 8-fold by IL-1β treatment 15-fold by TNFα treatment, and 80-fold by treatment with a combination of IL-1β, TNFα, and IL-6 (Fig 3A). Other cytokines, i.e. IL-6 or IL-8, did not affect IL-1β gene expression (Fig 3A). TNFα gene expression was enhanced 3-fold by IL-8 treatment, 3-fold by CCL20 treatment, 2-fold by IL-1β treatment, 3-fold by TNFα treatment, and 3.5-fold by treatment with a combination of IL-1β, TNFα, and IL-6 (Fig 3B). Addition of IL-17

did not affect TNFα gene expression (Fig 3B). Gene expression of IL-6 and IL-8 was effected by exogenous cytokine and chemokine treatment for 7 days, but not by RA-serum. IL-6 gene expression was enhanced 4-fold by CCL20 treatment, 3-fold by IL-17 treatment, 32-fold by IL-1β treatment, 25-fold by TNFα treatment, and 87-fold by treatment with a cocktail of IL-1β, TNFα, and IL-6 (Fig 4A). Treatment with IL-8 or IL-6 did not affect IL-6 gene expression at day 7 (Fig 3C). IL-8 gene expression was enhanced 7-fold by IL-8, 5-fold by CCL20 treatment, 24-fold by IL-1β treatment, 58-fold by TNFα treatment, and 233-fold by treatment with a combination of IL-1β, TNFα, and IL-6 (Fig 3D). RA-serum treatment enhanced the expression of IL-1β mRNA by 3.3-fold and expression of TNFα mRNA by 2.6-fold at day 7, but did not affect IL-6 and IL-8 mRNA expression by osteocytes (Fig 3E).

None of the cytokines or chemokines tested affected RANKL and OPG gene expression (Fig 4A, 4B). Only a combination of IL-1β, TNFα, and IL-6 treatment enhanced OPG gene expression by 2.6 fold (Fig 4B). RA-serum treatment did not affect RANKL and OPG gene expression by osteocytes at day 7 (Fig 4C).

Exogenous cytokine treatment affects FGF23 gene expression by osteocytes

The proteins FGF23 and MEPE play a role in phosphate homeostasis and are produced by osteocytes. MEPE, FGF23, and DAMP1 mRNA is expressed by osteocytes at day 7. None of the cytokines and chemokines tested affected MEPE gene expression (Fig 5A). FGF23 gene expression was enhanced 2.6-fold by IL-8 treatment 2.6-fold by CCL20 treatment, 2.3-fold by IL-1β treatment, 2.9-fold by TNFα treatment, and 3.9-fold by a combiination of IL-1β, TNFα, and IL-6 (Fig 5B). None of the cytokines affected DMP1 gene expression (Fig 5C). Only addition of the combination of IL-1β, TNFα, and IL-6 inhibited DMP1 gene expression by 2.2-fold (Fig 5C). RA-serum treatment did not affect MEPE, FGF23, and DMP1 gene expression by osteocytes (Fig 5D).

RA-serum and exogenous cytokine treatment affect Wnt signaling by osteocytes

The proteins SOST and DKK1 are produced by osteocytes and inhibit bone formation by inhibiting the stimulatory effect of Wnts on osteoblast differentiation. RA-serum, IL-1β, and a combination of IL-1β, TNFα, and IL-6 treatment affected Wnt signaling by altering SOST and DKK1 gene expression by osteocytes. SOST gene expression was enhanced 4.7-fold by IL-1β, and 8.5-fold by treatment with a combination of IL-1β, TNFα, and IL-6 (Fig 6A). IL-8, CCL20, IL-17, IL-6, or TNFα did not affect SOST gene expression (Fig 6A). None of the cytokines and chemokines tested affected DKK1 gene expression (Fig 6B). RA-serum treatment enhanced SOST gene expression by 2.5-fold and DKK1 expression by 2-fold (Fig 6C).

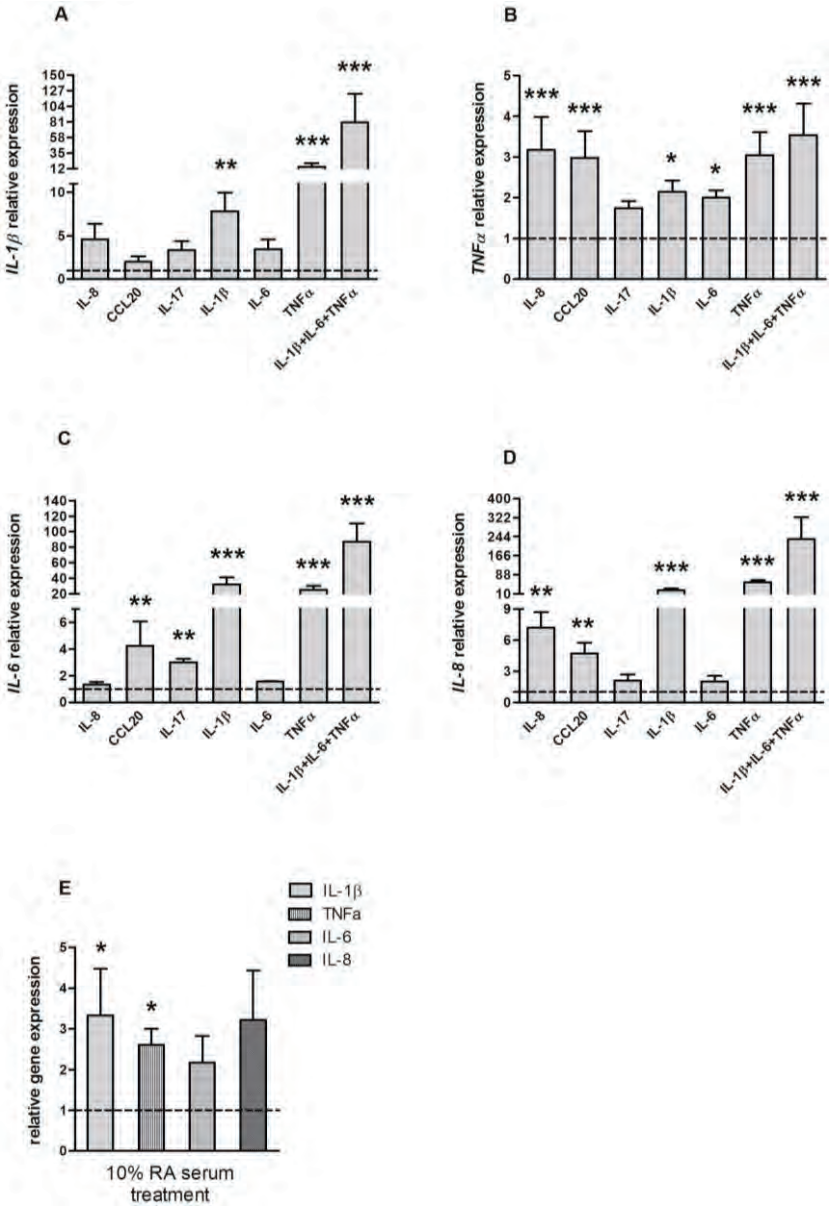

Figure 3. Effect of inflammatory cytokines, chemokines, a combination of cytokines, and RA-serum on IL-1β, TNFα, IL-6, and IL-8 gene expression by osteocytes after 7 days of culture. (A) IL-1β gene expression was enhanced by IL-1β and TNFα, but not IL-8, CCL20, IL-17, or IL-6. IL-1β gene expression was synergistically enhanced by the combination of IL-1β, TNFα, and IL-6. (B) TNFα gene expression was enhanced by IL-8, CCL20, IL-1β, IL-6, TNFα, and a combination of IL-1β, TNFα, and IL-6. (C) IL-6 gene expression was enhanced

by CCL20, IL-17, IL-1 β, TNFα, and a combination of IL-1β, TNFα, and IL-6. (D) IL-8 gene expression was enhanced by IL-8, CCL20, IL-1 β, TNFα, and a combination of IL-1β, TNFα, and IL-6. (E) RA-serum enhanced IL-1β and TNFα gene expression, but did not affect IL-6 and IL-8 gene expression. Values are mean±SEM from 6 independent experiments. Bone biopsies were obtained from 6 donors. Significant effect of cytokine, chemokine, a combination of cytokines, or RA-serum, *p<0.05, **p<0.01, ***p<0.001.

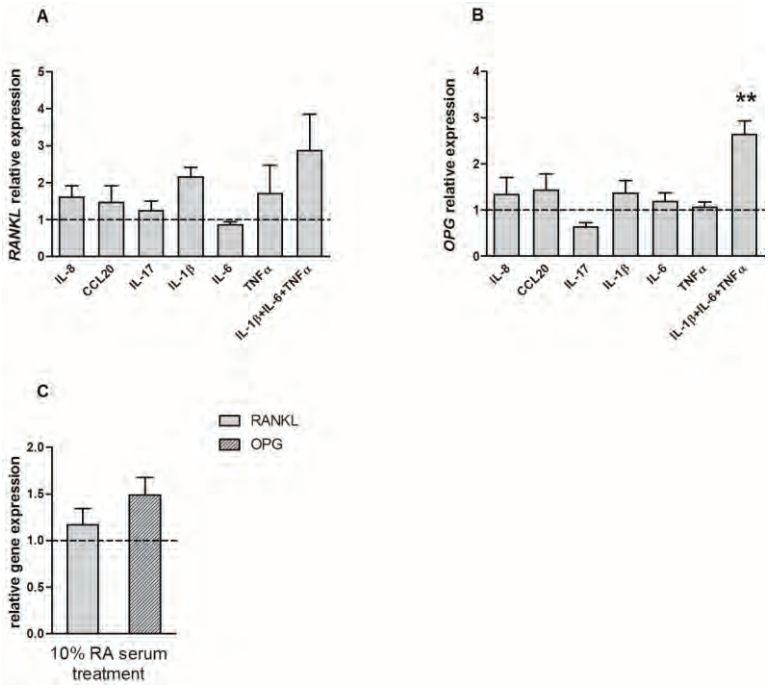

Figure 4. RANKL and OPG gene expression was not affected by inflammatory cytokines or RA-serum in osteocytes after 7 days of culture. (A) RANKL gene expression was not affected by IL-8, CCL20, IL-17, IL-1β, IL-6, TNFα, or a combination of IL-1β, IL-6, and TNFα. (B) OPG gene expression was not affected by IL-8, CCL20, IL-17, IL-1β, IL-6, or TNFα, but was enhanced by a combination of IL-1β, IL-6, and TNFα. (C) RA-serum did not affect RANKL or OPG gene expression by osteocytes. Values are mean±SEM from 6 independent experiments. Bone biopsies were obtained from 6 donors. experiments. Significant effect of cytokine, chemokine, a combination of cytokines, or RA-serum, **p<0.01.

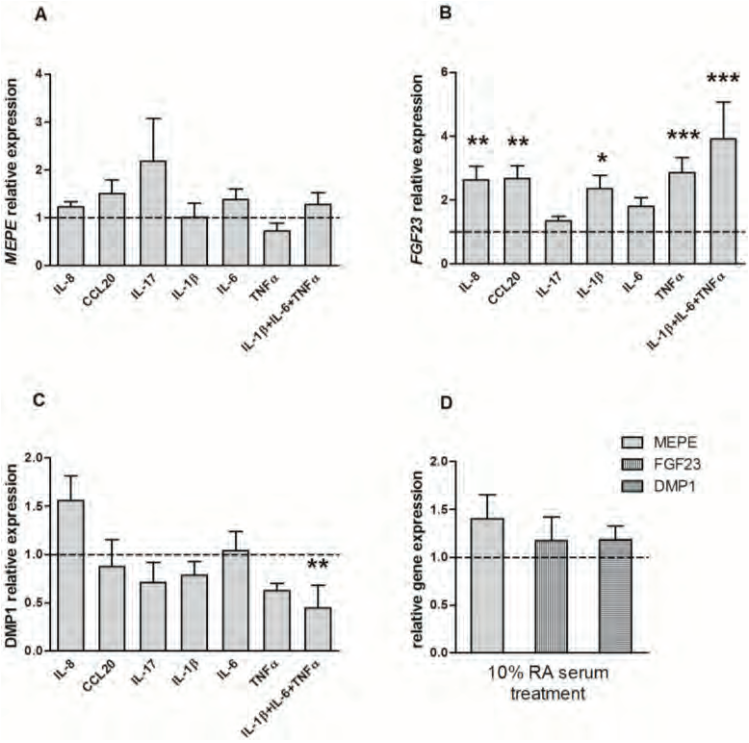

Figure 5. Effect of inflammatory cytokines, chemokines, a combination of cytokines, and RA-serum on gene expression of phosphate homeostasis-related signaling factors in osteocytes after 7 days of culture. (A) MEPE gene expression was not affected by IL-8, CCL20, IL-17, IL-1β, IL-6, TNFα, or a combination of IL-1β, IL-6, and TNFα. (B) FGF23 gene expression was enhanced by IL-8, CCL20, IL-1β, TNFα, and a combination of IL-1β, IL-6, and TNFα. (C) DMP1 gene expression was not affected by any cytokine or chemokine tested, but it was inhibited by a combination of IL-1β, IL-6, and TNFα. (D) MEPE, FGF23, and DMP1 gene expression was not affected by RA-serum. Values are mean±SEM from 6 independent experiments. Bone biopsies were obtained from 6 donors. Significant effect of cytokine, chemokine, a combination of cytokines, or RA-serum, *p<0.05, **p<0.01, ***p<0.001.

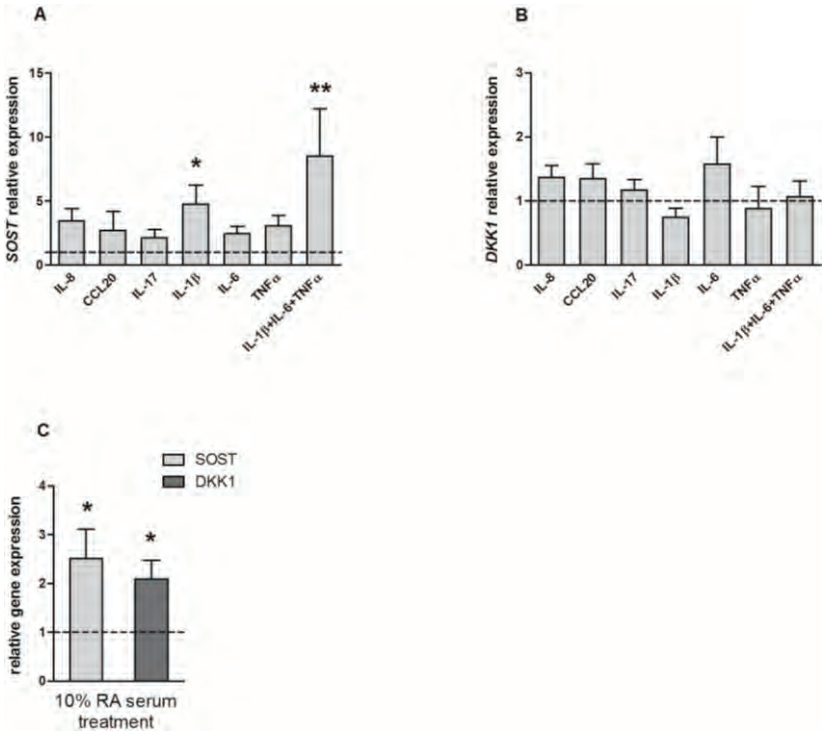

Figure 6. Effect of inflammatory cytokines, chemokines, a combination of cytokines, and RA-serum on gene expression of Wnt-signaling inhbitors in osteocytes after 7 days of culture. (A) SOST gene expression was enhanced by IL-1β and a combination of IL-1β, IL-6, and TNFα. (B) DKK1 gene expression was not affected by IL-8, CCL20, IL-17, IL-1β, IL-6, TNFα, or a combination of IL-1β, IL-6, and TNFα. (C) RA-serum enhanced SOST and DKK1 gene expression. Values are mean±SEM from 6 independent experiments. Bone biopsies were obtained from 6 donors. Significant effect of cytokine, chemokine, a combination of cytokines, or RA-serum, *p<0.05, **p<0.01.

DISCUSSION

In this study the effect of exogenous recombinant cytokines, chemokines, a combination of cytokines, and serum of patients with active RA on the expression of osteocyte singling molecules by bone cells cultured in close contact with their native matrix was investigated. We demonstrated that osteocytes cultured in their native matrix did express mRNA of osteocyte-specific signaling molecules, which are hardly expressed by primary bone cells cultured on tissue culture plastic, and their expression was altered by treatment with exogenous recombinant cytokines, chemokines, a combination of cytokines, and active RA-serum. Exogenous recombinant cytokines and treatment with a combination of cytokines enhanced IL-1β, TNFα, IL-6, IL-8, FGF23, and SOST gene expression, while active RA-serum treatment enhanced IL-1β, TNFα, SOST, and DKK1 gene expression. Our findings revealed that addition of inflammatory factors did alter osteocyte signaling *in vitro*, which might have implications for bone loss *in vivo*. This suggests that osteocytes could provide a new target to prevent inflammation-induced osteocyte-mediated bone loss.

Osteocytes cultured in their native matrix did express SOST, DMP1, FGF23, MEPE, DKK1, IL-1β, TNFα, IL-6, IL-8, RANKL, and OPG mRNA, which is in accordance with published data on the production of signaling molecules DMP1, MEPE, FGF23, SOST, OPG, and RANKL by osteocytes (15-19). We visualized the live osteocytes in cultured bone chips, and found that these osteocytes produced high amounts of sclerostin protein. So far only a few osteocyte cell lines established from chicken and mouse bone are available, and culture of human osteocytes *in vitro* remains a challenge. Therefore culture of denuded human bone chips with osteocytes embedded in their native matrix could overcome this challenge and provide a 3D model which can be used to study osteocyte function and signaling.

Not much is known about the effect of individual inflammatory cytokines, chemokines or a combination of inflammatory cytokines on gene expression of a panel of inflammatory cytokines, i.e. IL-1β, TNFα, IL-6, and IL-8 by osteocytes. In this study, we found that most of the individual exogenous cytokines and chemokines tested did enhance the expression of IL-1β, TNFα, IL-6, and IL-8 by osteocytes. However, IL-6 expression was not affected by IL-6 treatment of osteocytes. Kulkarni and colleagues reported that IL-6 treatment enhanced IL-6 expression by the mouse osteocyte cell line MLO-Y4 (18). This discrepancy might be related to the cell type and culture system used, i.e. we cultured human osteocytes embedded in their native matrix, while Kulkarni and colleagues cultured a mouse osteocyte cell line on a plastic substratum (18). Interestingly, a synergistic upregulation of IL-1β, IL-6, and IL-8 gene expression in osteocytes was observed by addition of a combination of IL-1β, TNFα, and IL-6. In addition, diluted (1:10)

active RA-serum enhanced gene expression of IL-1β and TNFα by osteocytes. These findings might explain the occurrence of local amplification of inflammation in bone during RA, thereby disturbing bone homeostasis. Human bone contains a high number of osteocytes (around 1.81 billion) (29). Our findings showing that osteocytes can produce inflammatory cytokines and signaling molecules, that are upregulated by adding inflammatory cytokines and active RA-serum, contribute to the limited knowledge currently available on the osteocyte as a modulator of inflammation.

Osteocyte-produced RANKL is essential for osteoclast formation and activity (21, 30). The inflammatory cytokines IL-1β and IL-6 enhance RANKL production in the mouse osteocyte cell line MLO-Y4 (18, 26). However, we found that exogenous cytokines, chemokines, and active RA-serum did not affect gene expression of RANKL and OPG by osteocytes. This difference might be due to the fact that we cultured human osteocytes in their native matrix, while Kulkarni et al. and Bakker et al. cultured the MLO-Y4 cells on plastic (18, 26).

Osteocytes not only produce cytokines, but also signaling molecules such as DMP1, MEPE, and FGF23, that are involved in matrix mineralization and phosphate homeostasis (14, 24, 31, 32). We found that osteocytes cultured in their native matrix highly express DMP1, MEPE, and FGF23 mRNA, which is hardly expressed by osteocytes cultured on plastic. MEPE and DMP1 gene expression was not affected by IL-8, CCL20, IL-17, IL-1β, IL-6, and TNFα treatment of osteocytes. Only the combination of IL-1β, IL-6, and TNFα inhibited DMP1 gene expression. However, TNFα has also been shown to inhibit DMP1 gene expression by human osteocytes cultured in their native matrix (17). This discrepancy might be related to the duration of TNFα treatment and the serum used; we treated osteocytes with TNFα for 7 days in 10% human serum, while Ito et al. treated osteocytes with TNFα for 24 h in 2% fetal clone serum (17). We found that FGF23 gene expression by osteocytes was enhanced by IL-8, CCL20, IL-1β, TNFα, and a combination of IL-1β, TNFα, and IL-6. Ito et al. found that IL-1β, TNFα, and lipopolysaccharides, but not IL-6 enhance FGF23 expression by osteocytes (17). We found that active RA-serum did not affect gene expression of MEPE, DMP1, or FGF23 by osteocytes, which might be due to the usage of diluted (1:10) active RA-serum, that lowers the concentration of inflammatory factors present. Elevated circulating levels of FGF23 cause hypophosphatemia and impaired bone mineralization (14, 24, 31, 32). Our results showing that the inflammatory cytokines IL-8, CCL20, IL-1β, and TNFα enhanced FGF23 gene expression, suggest that systemic inflammation might impair bone mineralization and phosphate homeostasis thereby affecting bone homeostasis in RA.

Osteocytes play a role in mechanotransduction, systemic bone mineral homeostasis, and signaling towards osteoclasts, as well as in signaling towards osteoblasts thereby affecting osteoblast differentiation and activity (13, 14, 33).

Osteocytes produce the proteins sclerostin and DKK1, which directly decrease osteoblast differentiation and bone matrix formation via an inhibition of the Wnt signaling pathway (34-36). TNFα enhances sclerostin and DKK1 production resulting in bone loss, while blocking of DKK1 reverses this bone loss in mice (37). We found that IL-1β, a combination of IL-1β, TNFα, and IL-6, and active RA-serum treatment enhanced SOST gene expression by osteocytes. Active RA-serum also enhanced DKK1 gene expression by osteocytes. These findings indicate that inflammatory cytokines during systemic inflammation inhibit osteoblast differentiation and activity via upregulation of sclerostin and DKK1 production by osteocytes.

Based on published data, we have chosen the cytokines IL-8, CCL20, IL-17, IL-6, IL-1β, and TNFα as possible players that could modulate osteocyte signaling (8, 9, 11, 38). We also tested whether there was a possible synergistic effect of the well-known potent inflammatory cytokines IL-1β, IL-6, and TNFα on osteocyte signaling. To mimic the *in vivo* situation during bone remodeling in active RA, we tested the effect of active RA-serum from a well-characterized patient group on osteocyte signaling using human osteocytes embedded in their native matrix. Live bone cells were visualized on the surface of bone chips after 7 days of culture, but these cells did not produce sclerostin or DKK1 as shown by immunohistochemistry. Since the bone chips were denuded by collagenase-2 treatment, these cells might be osteocytes that have crawled out from newly formed osteoid. Further research is needed to characterize the phenotypic character of the cells that appeared on the bone chip's surfaces after 7 days of culture. A limitation of this study might be the relatively low number of patients included. Statistical significance between groups was not easily obtained due to the fairly large data variation, probably due to donor variation.

In conclusion, active RA-serum, individual exogenous recombinant cytokines, chemokines, and a combination of cytokines modulated gene expression of cytokines, mineralization-related signaling molecules, phosphate homeostasis-related signaling molecules, and Wnt inhibitors in osteocytes. Therefore osteocytes could be a new target to prevent inflammation-induced osteocyte-mediated bone loss in inflammatory diseases.

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CHAPTER 8

General Discussion

GENERAL DISCUSSION

Bone is a dynamic and active tissue that under normal physiological conditions is continuously renewed by a process called bone remodeling. It consists of organic components such as collagen fibers and non-collagenous proteins, and inorganic components such as mineral salts. The bone remodeling process is executed by bone resorbing osteoclasts and bone depositing osteoblasts, which are orchestrated by mechanosensing osteocytes. Bone lining cells cover inactive (non-remodeling) bone surfaces, and may be involved in the propagation of the activation signal that initiates bone resorption and bone remodeling. Osteoblasts and osteocytes are derived from mesodermal progenitors residing in periost and in the bone marrow, or blood vessel associated precursor cells. Osteoclasts arise from hematopoietic cells of the monocyte/macrophage lineage. Physiological concentrations of cytokines and signaling molecules are essential for the bone remodeling process, while altered cytokine and signaling molecule levels disturb bone homeostasis (1-3).

Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown etiology, characterized by polysynovitis with painful and swollen joints, local bone erosion, joint space narrowing, and extra-articular manifestations such as generalized osteoporosis (4, 5). RA is a common disease affecting around 1% of the human population worldwide (6, 7). The prevalence is three times higher in women compared to men (8, 9). The exact etiology of RA is still unclear, but some environmental and genetic factors predetermine the risk of developing RA and the severity of this disease (10, 11). The presence of the human leukocyte antigen (HLA)-DRB1 or HLA-DR4 haplotype, a single-nucleotide polymorphism in the gene protein tyrosine phosphatase non-receptor type 22 (PTPN22), and the presence of anti-cyclic citrullinated peptide (anti-CCP) antibodies are associated with development of RA (11-15). Other factors associated with RA are age, hormonal factors, and lifestyle factors such as higher body mass index (BMI) and smoking (9, 13-15).

In RA, lymphocytes and macrophages infiltrating the synovium cause inflammation and pannus formation (4, 16). Pannus is a synovial membrane-derived tissue expanding on and invading into cartilage and the underlying bone matrix. The leading edge of pannus is composed of fibroblast- and macrophage-like cells, which produce proteinases able to cause destruction of articular cartilage. Cooperation of mesenchymal cells and macrophage-like progenitor cells leads to local inflammatory cytokine production and formation of osteoclasts, that invade the subchondral bone using acid attack and acidic proteinases. Pannus invasion leads to cartilage and bone erosions around the inflamed joints. Cells in pannus in RA produce high amounts of cytokines, chemokines, as well as signaling molecules, that can easily enter the circulation and reach the bone cells (17-19).

Inflammatory cytokines such as interleukin-1, beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) have autocrine, paracrine, and endocrine effects on different cell types in the body (3, 17). Focal articular, periarticular, and generalized bone loss in the appendicular and axial skeleton is frequently observed in patients with RA (4, 5). Early stage RA patients show decreased bone mineral density, a 2-fold increase in risk of vertebral and non-vertebral (including hip) fractures compared with healthy controls (20-23). Such fractures are associated with severe morbidity and increased mortality rate (24). Therefore generalized osteoporosis represents a serious side effect of RA.

Synovial inflammation results in focal erosion of articular bone and cartilage, and synovial fluid contains highly elevated levels of inflammatory cytokines, chemokines, and signaling molecules (25). The inflammatory cytokines present in inflamed synovium enhance the recruitment, differentiation, and activation of osteoclasts and inhibit differentiation of osteoblasts thus causing local bone loss (26-28). However the causes of generalized bone loss in RA remain unclear. Generalized osteoporosis in RA is in part caused by immobility and corticosteroid therapy (29, 30), but it has also been attributed to the effects of systemic inflammation, such as elevated levels of circulating cytokines, chemokines, and signaling molecules. In postmenopausal women estrogen deficiency induces bone loss by upregulating cytokine production in immune cells (31). As a result of the aging process, the bone deteriorates in composition, structure, and function, which predisposes to osteoporosis (32). Bone loss in aging is also related to low-grade chronic inflammatory status caused by an increased prevalence of a clustering of metabolic abnormalities with an inflammatory pathogenesis including obesity, dyslipidemia, hypertension, insulin resistance, and type 2 diabetes (32, 33). Therefore in the aging population with RA, fracture risk related to generalized osteoporosis might increase.

RA-serum is a complex mixture of cytokines, biological proteins and signaling molecules such as receptors, receptor antagonists, antibodies, and autoantibodies. Therefore the cumulative effect of all these factors on bone homeostasis might be different from the effect of an individual inflammatory cytokine. The levels of cytokines and chemokines in the circulation are lower compared with the levels in synovial fluid in RA. However a complex combination of inflammatory cytokines, chemokines, and signaling factors present in the circulation of patients with active RA may certainly be sufficient to locally trigger inflammatory cytokine production within bone in a positive feedback loop, which might amplify inflammation in the bone remodeling unit, and disturb bone homeostasis. Failure of synthetic disease-modifying antirheumatic drugs (DMARDs) and biological DMARDs to mitigate inflammation and bone loss is still experienced in the clinic in too many RA patients. Therefore more effective treatment approaches, such as a remission-induction strategy in early RA (34), but

also additional therapeutic agents for the therapy-resistant patients to reduce inflammation and bone loss are still needed. A complete picture of the factors and mechanisms involved in inflammation-induced bone loss is required to discover such a new therapeutic agent. So far the effect of a complex combination of inflammatory cytokines, chemokines, and signaling factors present in the circulation of patients with systemic inflammation on bone homeostasis is unknown. To understand the mechanisms involved in inflammation-induced altered bone homeostasis in RA, we tested the effects of serum from patients with active RA on bone cell formation, function, and communication *in vitro* by adapting the research methods as described in **chapter 2**.

Endochondral ossification is a crucial process during fracture healing and critical size bone defect repair (35-37). An acute inflammatory phase is necessary to initiate endochondral ossification (38, 39). Continuous treatment by IL-1β or TNFα however inhibits the chondrogenic phase of endochondral ossification (28). Moreover, complications in bone healing including delayed fracture healing have been reported during systemic inflammation (40-42). In **chapter 3**, we investigated the effect of active RA-serum on osteochondrogenic differentiation of two different precursor cell types, i.e. ATDC5 cells and human periosteum-derived mesenchymal stem cells (HPDC), mimicking systemic inflammation as occurs during the active disease stage of RA. We found that serum from patients with active RA inhibits differentiation of osteochondrogenic precursor cells, as shown by decreased cartilage matrix accumulation and changes in osteochondral gene expression. Such an inhibition may explain, at least in part, the decrease in bone formation and delayed fracture healing in patients with RA, since inflammatory cytokines and signaling molecules present in the circulation of patient with systemic inflammation affect differentiation of mesenchymal stem cells towards endochondral ossification. Less bone formation and less bone healing decrease bone mass causing osteoporosis, which increase the fracture risk even more in RA patients.

Various growth factors and inflammatory cytokines including IL-1β and TNFα affect osteoblast differentiation and bone formation (1-3). However it is still unclear whether systemic inflammation inhibits osteoblast differentiation and osteoblast function leading to low bone mass. In **chapter 4** we studied whether a complex mixture of circulating inflammatory mediators present in the serum of patients with active RA alters osteoblast function compared with serum from the same patients in clinical remission. We demonstrated that active RA-serum inhibits osteoblast proliferation and differentiation. These results indicate that the circulation of patients with active RA contains factors that inhibit osteoblast proliferation and differentiation, which is not the case when they are in remission. Such an osteoblast inhibition might cause less bone formation in patients with active RA. This might lead to generalized osteoporosis and increased fracture risk

in these patients, and possibly also in patients with other systemic inflammatory disorders.

The serum of patients with active RA might not only contain inflammatory cytokines such as IL-1β, IL-6, interleukin-17 (IL-17), and TNFα, but also growth factors, their inhibitors, and chemokines (18, 19, 43, 44). DKK1 inhibits the Wnt signaling pathway resulting in diminished osteoblast formation and function (45). The inflammatory cytokines IL1β, IL-6, IL17, and TNFα inhibit osteogenic differentiation thereby affecting bone remodeling (3, 17, 27, 46). However the effect of chemokines on osteoblast proliferation and differentiation is still unclear. In **chapter 5** we analyzed whether the chemokines CXCL8 and CCL20 potentially affect osteoblast proliferation and differentiation, since elevated levels of these chemokines are found in RA serum. Elevated levels of the chemokines CXCL9 and CXCL10 were also found in RA serum, but their receptors were not detected in human primary osteoblasts. We found that CXCL8 and CCL20 did not inhibit osteoblast proliferation nor gene expression of the main matrix proteins collagen1 (COL1), osteopontin (OPN), and osteocalcin (OCN). However, CXCL8 and CCL20 might have an effect on bone homeostasis via other mechanisms as discussed below.

The primary function of osteoblasts is to form bone, but they also release cytokines and signaling molecules that play a crucial role in bone homeostasis (47, 48). The inflammatory cytokines IL-1β and TNFα affect the production of cytokines and signaling factors by osteoblasts (47). We investigated whether circulating inflammatory mediators present in the serum of patients with active RA alter cytokine production by osteoblasts and/or osteoblast-mediated osteoclastogenesis compared with serum from the same patients in clinical remission (**chapter 4**). We found that active RA-serum enhanced IL-6 and Receptor activator of nuclear factor kappa-B ligand (RANKL) gene expression in osteoblasts, as well as osteoblast-mediated osteoclastogenesis. Blocking of IL-6 and RANKL in co-cultures of RA-serum–pretreated osteoblasts and osteoclast precursors reduced the number of osteoclasts. Moreover, CXCL8 and CCL20 also affected osteoblast-mediated osteoclastogenesis partly via IL-6 production by osteoblasts (**chapter 5**). These results indicate that a complex combination of inflammatory factors, including the chemokines CXCL8, CCL20, in the circulation can disturb bone homeostasis in RA by altering osteoblast-to-osteoclast communication. Elevated levels of CXCL8 and CCL20 are present in the circulation of patients with active RA. We found that not only active RA-serum, but also the individual chemokines CXCL8 and CCL20 enhanced osteoblast-mediated osteoclastogenesis. Moreover CCL20 enhanced osteoblast-mediated osteoclastogenesis and osteoclast activity. Molecular and cellular heterogeneity in RA impacts clinical outcome to therapies targeting different biological pathways (49). Analysis of gene-set modules and serum biomarkers suggests differential clinical response to anti-TNFα and anti-IL6R

therapy, which is dependent in part on the presence of B cell-dominated or inflammatory macrophages and NF-κB-activating cytokines-dominated axes (49). Correlation of personalized gene expression of cytokines, chemokines, and other signaling molecules with the response to biological treatment in RA patients might help to choose an appropriate biological drug to treat the disease (50). Therefore CXCL8 and/or CCL20 could be (a) possible new candidate(s) in gene expression signature-based personalized medicine for RA patients in the future.

Osteocytes are highly mechanosensitive; after mechanical stimulation they alter the production of a range of signaling molecules, which modulate recruitment, differentiation, and activity of osteoblasts and osteoclasts (51-56). Therefore the cytokines and signaling molecules produced by osteocytes play a crucial role in bone adaptation (55-57). Osteocytes also play an important role in phosphate homeostasis via dentin matrix protein 1(DMP1), Phosphate-regulating neutral endopeptidase (PHEX), Matrix extracellular phosphoglycoprotein (MEPE), and fibroblast growth factor 23 (FGF23) (56, 58, 59). Osteocytes produce sclerostin and dickkopf WNT signaling pathway inhibitor 1 (DKK1), that decrease bone formation by inhibiting the stimulatory effect of Wnts on osteoblast differentiation and activity (45, 60). Osteocytes communicate with kidney, parathyroid glands, and muscle via FGF23 production under influence of MEPE (61, 62). Moreover osteocytes communicate with muscle via production of insuline-like growth factor (IGF-1) and mechano growth factor (MGF) (63). Therefore osteocytes act asan endocrine organ rather than just as bone cells. Inflammatory cytokines IL-1β and TNFα reduce the intrinsic capacity of osteocytes to sense mechanical stimuli (64). However it is unknown whether a complex combination of cytokines and signaling factors present in active RA-serum affects osteocyte mechanosensitivity. In **chapter 6**, we describe that active RA-serum did not affect the intrinsic capacity of osteocytes to sense mechanical stimuli, i.e. the osteocytes showed an unchanged NO response as well as unaltered IL-6, cyclooxygenase 2 (COX2), cysteine rich protein 61 (CYR61), MEPE, and SOST gene expression response to pulsating fluid flow (PFF), although prostaglandin E_2 (PGE_2) production was affected by PFF. Determination of the exact concentration of inflammatory cytokines present in the circulation of patients with active RA that reach the osteocytes is difficult. However, the effect of active RA-serum on osteocyte mechanosensitivity *in vivo* might be even higher than can be extrapolated from our *in vitro* findings, since we used diluted (1:10) RA-serum, which lowers the concentration of cytokines and signaling factors.

Treatment of osteocytes with the inflammatory cytokine IL-1β or TNFα stimulates osteocytic cytokine and signaling molecule production (46, 58, 65). Interestingly, mechanical loading prevents the stimulating effect of IL-1β on osteocyte-mediated osteoclastogenesis (65). In **chapter 6**, we investigated whether active RA-serum affects osteocyte-to-osteoclast communication, and

whether mechanical loading of osteocytes can alter this effect. We found that active RA-serum enhanced the RANKL/OPG gene expression ratio by osteocytes as well as osteocyte-mediated osteoclastogenesis, and that mechanical loading of osteocytes reversed these effects. These results are in accordance with the findings of Kulkarni and colleagues (65). Our data indicate that systemic inflammation in RA could cause generalized bone loss by enhancing osteocyte-to-osteoclast communication, and that mechanical stimulation of osteocytes may prevent such bone loss. Moreover mechanical loading suppresses the catabolic effects of IL-1β in fibrochondrocytes from the temporomandibular joints (66). Therefore physical exercise, which can be performed by RA patients without pain, or other forms of bone loading e.g. vibrating platforms, could thus have therapeutic potential in the prevention of osteoporosis in RA and other inflammatory diseases. In **chapter 7** we investigated the effect of exogenous recombinant inflammatory cytokines and active RA-serum containing a combination of inflammatory cytokines and signaling molecules on human osteocyte signaling. We found that exogenous recombinant inflammatory cytokines enhanced gene expression of IL-1β, IL-6, IL-8, TNFα, FGF23, and SOST by osteocytes. Similarly, active RA-serum enhanced gene expression of IL-1β, TNFα, FGF23, SOST, and DKK1 by osteocytes, which is in accordance with the findings by others (58, 60, 65, 67, 68). These results suggest that osteocytes might play a key role in bone loss during systemic inflammation, and therefore could represent a new target to prevent bone loss in inflammatory diseases. Sclerostin and DKK1 are known to decrease osteoblast differentiation by inhibiting Wnt signaling. Culture of mesenchymal stem cells with conditioned medium from osteocyte cultures treated with cytokines or active RA-serum with or without blocking Wnt inhibitors sclerostin and DKK1 could provide more insight on inflammation-mediated osteocyte-to-osteoblast communication.

Proper primary *human* osteocytes and *human* osteocyte cell lines for *in vitro* research are not available so far. The outgrowth human bone cells from denuded human iliac crest trabecular bone chips have been shown to exhibit both an osteocyte and an osteoblast-like phenotype and are more mechanosensitive than osteoblast cell lines and fibroblasts (69-72). Therefore, we used these bone cells as a model for osteoblasts in **chapters 4** and **5**, and as a model for osteocytes in **chapter 6**. The effects of active RA-serum in the heterogeneous osteoblast/osteocyte population used might not be as prominent as when the effects would have been tested in a homogeneous osteoblast or osteocyte population. Therefore the limitation of the use of the heterogeneous osteoblast/osteocyte population might only underestimate the real effect of RA-serum on osteoblasts and/or osteocytes. Another limitation of this study might be the relatively low number of RA patients included. Statistical significance between groups is not easily obtained due to the fairly large data variation, probably due to donor variation and the low number of patients included. A strength of our study is

that we used a very well-defined RA patient group. The RA-serum used in **chapters 3** and **4** was collected from the same patients at two time points with a one-year interval. The first time point was during the early stage of the disease (less than 1 year disease duration) and before the patients had taken DMARDs or corticosteroids, and the second time point after remission of disease. Corticosteroid therapy was stopped 3 months before serum collection during remission, thereby avoiding a possible effect of corticosteroids on bone cells. Such an experimental set up gave us an opportunity to use the remission RA-serum from the same patient as a control, which reduces the inter-donor variation. We used human primary cells and human serum instead of animal cells or cell lines, which more closely resembles the *in vivo* situation in humans. Inflammatory cytokines are known to directly affect osteoclast formation and activity. Osteoclasts communicate with osteoblasts via production of factors, e.g. Transforming growth factor-beta (TGFβ), bone morphogenetic proteins (BMPs), Insulin-like growth factor II (IGF-II), ephirinB2, and connexins (73-75). In the present study, we did not address a direct effect of active RA-serum on osteoclast formation and activity, nor the effect of RA-serum on osteoclast-to-osteoblast communication. Therefore, future studies addressing these issues might provide more insight in inflammation-induced bone loss.

In conclusion, diluted (1:10) active RA-serum already affected osteoblast function, osteoblast-to-osteoclast communication, osteochondrogenic differentiation of precursor cells, osteocyte signaling, and osteocyte-to-osteoclast communication but not osteocyte mechanosensitivity. These findings suggest that the effects of a complex combination of inflammatory cytokines, chemokines, and signaling factors present in the circulation of patients with active RA on bone cell function and communication might be even more pronounced *in vivo*. This may affect bone homeostasis causing systemic bone loss and increasing fracture risk *in vivo* as illustrated in Figure 1. Mechanical stimulation of osteocytes was able to attenuate the active RA-serum–mediated enhanced osteocyte-to-osteoclast communication. Therefore, physical activity or other forms of bone loading, e.g. vibrating platform, could thus have therapeutic potential for generalized bone loss in RA and other inflammatory diseases. It is still unclear how a complex combination of cytokines and signaling factors present in active RA-serum causes bone loss. Therefore more research on the detection of the key factors and mechanisms in RA responsible for altered bone cell function and crosstalk between cells involved in bone homeostasis is needed. These studies might help to reveal the most critical cytokines, chemokines, and signaling factors involved in bone loss. This in turn might lead to the development of drugs for new targets and interventions, which might be more effective to mitigate inflammation-related bone loss than the already available targets. Such drugs can help to design more personalized treatments for generalized bone loss.

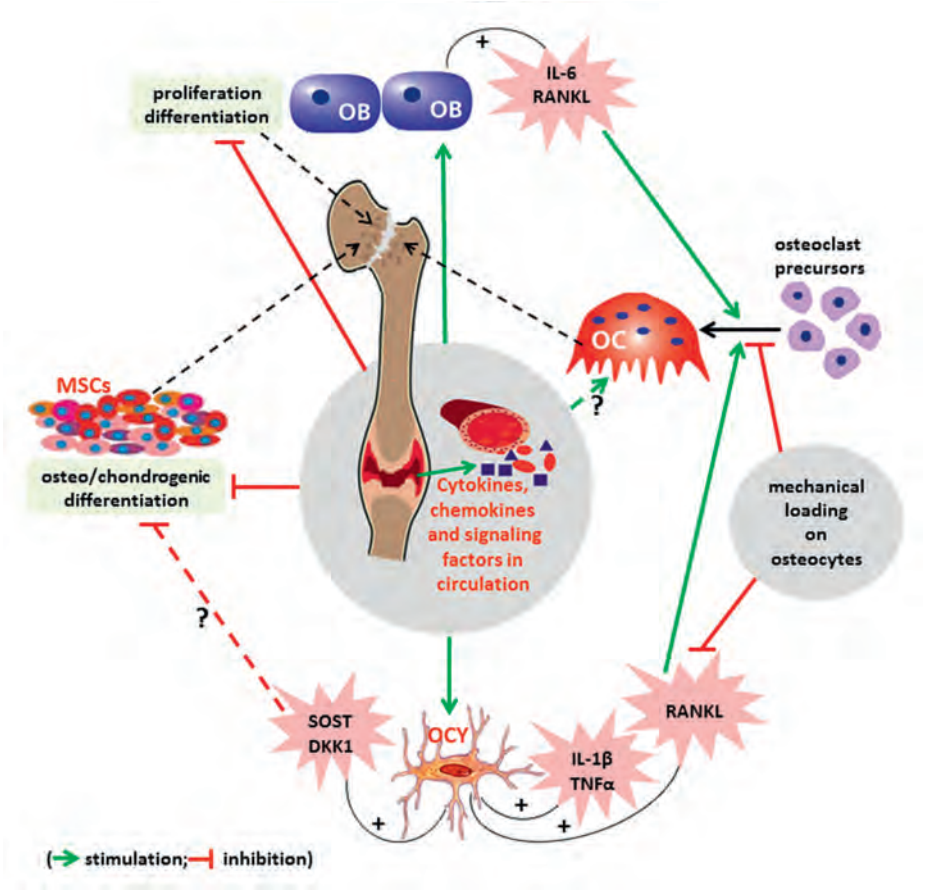

Figure 1. Mechanism through which bone homeostasis in the whole body skeleton could be affected by systemic inflammation in active RA. Immune cells recruited into the inflamed synovium produce inflammatory mediators, i.e. inflammatory cytokines, chemokines, and signaling factors. These inflammatory mediators can easily enter the blood circulation and reach the bone cells in the skeleton. They inhibit osteoblast proliferation and differentiation, and enhance osteoblastic IL-6 and RANKL production. IL-6 and RANKL stimulate osteoclastogenesis, which might facilitate bone loss. Inflammatory mediators inhibit osteo/chondrogenic differentiation of precursor cells, thereby affecting bone regeneration. They enhance the RANKL/OPG gene expression ratio by osteocytes, which stimulates osteoclastogenesis, while mechanical loading on osteocytes mitigates such effect. Inflammatory mediators affect osteocyte signaling via production of IL-1β, TNFα, SOST, and DKK1 by osteocytes. Sclerostin and DKK1 are inhibitors of osteoblast formation and function via inhibition of Wnt signaling. Consequently the net effect of a complex combination of inflammatory mediators present in the circulation of RA patients on bone cells causes generalized bone loss.
Abbreviations used: OB, osteoblast; OC, osteoclast; OCY, osteocyte; MSCs, mesenchymal stem cells.

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The Etiology of Generalized Osteoporosis in Rheumatoid Arthritis

General Summary

GENERAL SUMMARY

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects 0.5-1% of the world's population. Generalized osteoporosis is an extra-articular complication of RA that results in increased fracture risk and associated morbidity, mortality, and healthcare costs. The incidence of generalized osteoporosis among patients with rheumatoid arthritis is 15-20%. Generalized osteoporosis in RA is in part caused by immobility and corticosteroid therapy, but it has also been attributed to the effects of systemic inflammation, such as elevated levels of circulating cytokines, chemokines, and signaling molecules.

Serum of patients with active RA contains altered levels of cytokines, biological proteins, and signaling molecules such as soluble receptors, receptor antagonists, antibodies, and autoantibodies. Individual inflammatory cytokines are known to affect bone cell formation and function, thereby affecting bone homeostasis. However the effect of serum from patients with active RA (active RA-serum) containing a complex mixture of cytokines, chemokines, biological proteins, and signaling molecules, on bone cell formation, function, and communication is unknown. Therefore in this thesis we investigated the effect of active RA-serum on bone cell formation, function, and communication towards other cell types. In **chapter 2**, we describe the existing research methods that can be used to test the effects of active RA-serum on bone cell formation, function, and communication *in vitro*.

In **chapter 3**, we investigated the effect of active RA-serum on osteochondrogenic differentiation of two different precursor cell types, i.e. mouse chondrogenic ATDC5 cells and human periosteum-derived mesenchymal stem cells. We found that serum from patients with active RA inhibits differentiation of osteochondrogenic precursor cells, as shown by decreased cartilage matrix accumulation and changes in gene expression of bone and cartilage markers. Such an inhibition may explain, at least in part, the decrease in bone formation and delayed fracture healing in patients with RA, since inflammatory cytokines and signaling molecules present in the circulation of patients with RA affect differentiation of mesenchymal stem cells towards a bone or cartilage xenotype.

In **chapter 4**, we studied whether a complex mixture of circulating inflammatory mediators present in the serum of patients with active RA alters osteoblast function and communication towards osteoclast precursors compared with serum from the same patients in clinical remission. We demonstrated that active RA-serum inhibits osteoblast proliferation and differentiation. We also found that active RA-serum enhanced IL-6 and receptor activator of nuclear factor kappa-B ligand (RANKL) gene expression in osteoblasts, as well as osteoblast-mediated osteoclastogenesis. Blocking of IL-6 and RANKL in co-cultures of RA-serum–pretreated osteoblasts and osteoclast precursors reduced the number of

osteoclasts. Such an inhibition of osteoblast function and altered osteoblast-to-osteoclast communication by serum containing inflammatory mediators might explain the catabolic effect of systemic inflammation on bone mass.

Elevated levels of CXCL8 and CCL20 are found in RA-serum, but their effect on osteoblasts is unknown. Therefore, in **chapter 5,** we analyzed whether the chemokines CXCL8 and CCL20 potentially affect osteoblast proliferation and differentiation, and osteoblast-to-osteoclast communication. We found that CXCL8 and CCL20 did not inhibit osteoblast proliferation nor gene expression of the main matrix proteins. However, CXCL8 and CCL20 might have an effect on bone homeostasis via other mechanisms. We demonstrated that chemokines CXCL8 and CCL20 enhanced osteoblast-mediated osteoclastogenesis partly via IL-6 production by osteoblasts. Interestingly, these results correspond with data using RA-serum (**chapter 4**). Moreover conditioned medium from osteoblasts cultured with CCL20, but not CXCL8, enhanced osteoclast activity. Based on the results from **chapter 4 and 5**, we speculate that RA-serum-induced osteoblast-mediated osteoclastogenesis might be partly caused by CXCL8 and CCL20 present in active RA-serum. This qualifies these chemokines as interesting targets for future research on personalized medicine for RA patients who are not responding to the existing treatments.

Osteocytes are highly mechanosensitive; after mechanical stimulation they alter the production of a range of signaling molecules, that modulate recruitment, differentiation, and activity of osteoblasts and osteoclasts. Therefore the cytokines and signaling molecules produced by osteocytes play a crucial role in bone adaptation. In **chapter 6**, we investigated whether active RA-serum affects the intrinsic capacity of osteocytes to sense mechanical stimuli as well as osteocyte-to-osteoclast communication, and whether mechanical loading of osteocytes can alter the effect of RA-serum on osteocyte-to-osteoclast communication. We used primary human bone cells containing a mixture of osteoblasts and osteocytes like cells. Since osteocytes are more mechanoresponsive to mechanical stimuli than osteoblasts, we designated these cells “osteocytes” in **chapter 6**. We found that active RA-serum did not affect the intrinsic capacity of osteocytes to sense mechanical stimuli. We demonstrated that active RA-serum enhanced the RANKL/OPG gene expression ratio by osteocytes as well as osteocyte-mediated osteoclastogenesis, and that mechanical loading of osteocytes reversed these effects. These findings suggest that physical exercise, that can be performed by RA patients without pain, or other forms of bone loading, e.g. vibrating platforms, could have therapeutic potential for the prevention of osteoporosis in RA and other inflammatory diseases.

Activation of canonical Wnt signaling exclusively in osteocytes induces bone anabolism and triggers Notch signaling. Osteocytes have a function in phosphate homeostasis. For this purpose osteocytes produce a range of cytokines

and signaling molecules. In previous chapters, a mixture of osteoblast-like and osteocyte-like cells were used for experiments, but we would like to have a more pure culture of human osteocytes, preferably in their own environment. Unfortunately there is lack of a proper human osteocyte models to investigate their function *in vitro*. In **chapter 7**, we cultured human bone chips containing osteocytes embedded in their native matrix, and analyzed the effect of exogenous recombinant cytokines, chemokines, and active RA-serum on osteocyte signaling. We found that exogenous recombinant inflammatory cytokines enhanced gene expression of the cytokines IL-1β, IL-6, IL-8, TNFα, FGF23 which is involve in phosphate homeostasis, and Wnt antagonist SOST, while the chemokine CCL20 enhanced TNFα, IL-6, and IL-8 gene expression by osteocytes. Similarly, serum from patients with active RA enhanced gene expression of IL-1β, TNFα, FGF23,SOST, and Wnt antagonist DKK1 by osteocytes. These results suggest that osteocytes might play a key role in bone mass regulation during systemic inflammation, and therefore could represent an excellent target to prevent loss of bone mass in inflammatory diseases.

In this thesis we demonstrated that diluted (1:10) active RA-serum already affected osteoblast function, osteoblast-to-osteoclast communication, osteochondrogenic differentiation of precursor cells, osteocyte signaling, and osteocyte-to-osteoclast communication, indicating even more pronounced effects *in vivo*. Such effects may disturb bone homeostasis causing systemic bone loss and increasing fracture risk *in vivo*. Mechanical stimulation of osteocytes was able to attenuate the active RA-serum–mediated enhanced osteocyte-to-osteoclast communication, suggesting mechanical stimulation of osteocytes as a possible therapeutic option for bone loss in RA. The results described in this thesis contribute to opening a new research direction in osteoimmunology because failure of existing drugs to mitigate inflammation and bone loss are frequently experienced in the clinic in too many patients. This in turn might lead to the development of drugs for new targets and interventions such as antibodies against CXCL8 and CCL20, which might be more effective to mitigate inflammation-related bone loss when existing drugs are not effective. Such drugs can help to design more personalized treatments for generalized bone loss in systemic inflammation.

Het Onstaan van Osteoporose bij Patiënten met Reuma

Algemene Samenvatting

ALGEMENE SAMENVATTING

Reumatoïde artritis (RA) is een chronische ontstekingsziekte die voorkomt bij 0.5-1% van de wereldbevolking. Systemische osteoporose is een extra-articulaire complicatie van RA, die resulteert in een toename van het fractuurrisico en de daarmee gepaard gaande morbiditeit en mortaliteit, alsmede medische kosten. De incidentie van systemische osteoporose bij patiënten met RA is 15-20%. Systemische osteoporose bij RA wordt deels veroorzaakt door immobiliteit en behandeling met corticosteroïden, maar het wordt ook toegeschreven aan de gevolgen van systemische ontsteking, zoals verhoogde concentraties van circulerende cytokines, chemokines en signaalmoleculen.

Het serum van patiënten met actieve RA bevat veranderde concentraties van cytokines, eiwitten en signaalmoleculen, zoals oplosbare receptoren, receptor antagonisten, antistoffen en autoantistoffen. Het is bekend dat individuele inflammatoire cytokines de vorming van botcellen en hun functie beïnvloeden, waarbij de bothomeostase wordt aangedaan. Echter, het effect van serum van patiënten met actieve RA (actief RA-serum), dat een complex mengsel van cytokines, chemokines, eiwitten en signaalmoleculen bevat, op de vorming van botcellen, hun functie en communicatie is nog onbekend. Daarom hebben we in dit proefschrift onderzocht wat het effect is van actief RA-serum op de vorming van botcellen, hun functie en communicatie met andere celsoorten. In **hoofdstuk 2** beschrijven we de bestaande onderzoeksmethodes die gebruikt kunnen worden om de effecten van actief RA-serum op de vorming van botcellen, hun functie en communicatie *in vitro* te testen.

In **hoofdstuk 3** hebben we het effect bestudeerd van actief RA-serum op de osteochondrogene differentiatie van twee verschillende soorten voorlopercellen, te weten muizen chondrogene ATDC5 cellen en humane mesenchymale stamcellen afkomstig van het periosteum. Wij vonden dat het serum van patiënten met actieve RA de differentiatie van osteochondrogene cellen vermindert, hetgeen bleek uit een verminderde vorming van kraakbeenmatrix en veranderingen in de genexpressie van bot en kraakbeen merkstoffen. Een dergelijke remming zou tenminste gedeeltelijk kunnen verklaren waarom er sprake is van minder botvorming en vertraagde fractuurgenezing in patiënten met RA, omdat inflammatoire cytokines en signaalmoleculen in de circulatie van patiënten met RA van invloed zijn op de differentiatie van mesenchymale stamcellen tot bot of kraakbeen xenotype.

In **hoofdstuk 4** hebben we bestudeerd of een complex mengsel van circulerende inflammatoire mediatoren in het serum van patiënten met actieve RA de functie van osteoblasten en hun communicatie met osteoclast voorlopercellen verandert, in vergelijking met serum van dezelfde patiënten in klinische remissie. We konden aantonen dat serum van patiënten met actieve RA een remmende

werking heeft op de proliferatie en differentiatie van osteoblasten. Tevens vonden we dat serum van patiënten met actieve RA de genexpressie van IL-6 en receptor activator of nuclear factor kappa-B ligand (RANKL) stimuleerde, als ook de osteoblast-gemedieerde osteoclastogenese. Het blokkeren van IL6 en RANKL in co-culturen van osteoclast-voorlopercellen en osteoblasten, die waren voorbehandeld met serum van RA patiënten, reduceerde het aantal osteoclasten. Een dergelijke remming van de osteoblast functie en verandering in osteoblast-osteoclast communicatie door serum met inflammatoire mediatoren zou het katabole effect van systemische ontsteking op de botmassa kunnen verklaren.

Er worden verhoogde CXCL8 en CCL20 concentraties aangetroffen in RA-serum, maar het effect van deze chemokines op osteoblasten is niet bekend. Daarom hebben wij in **hoofdstuk 5** onderzocht of CXCL8 en CCL20 de proliferatie en differentiatie van osteoblasten en de osteoblast-osteoclast communicatie kunnen beïnvloeden. We zagen dat CXCL8 en CCL20 noch een remming veroorzaakten van de proliferatie van osteoblasten noch van de genexpressie van de belangrijkste matrixeiwitten. Echter, CXCL8 en CCL20 zouden via andere mechanismen een effect kunnen hebben op de bothomeostase. Wij konden aantonen dat de chemokines CXCL8 en CCL20 deels de osteoblast-gemedieerde osteoclastogenese stimuleerden via IL6 productie door osteoblasten. Het is interessant dat deze resultaten overeenkomen met data waarbij gebruik werd gemaakt van serum van RA patiënten (**hoofdstuk 4**). Daarnaast bleek geconditioneerd medium van osteoblasten gekweekt met CCL20, maar niet dat van osteoblasten gekweekt met CXCL8, de activiteit van osteoclasten te stimuleren. Gebaseerd op de resultaten uit **hoofdstuk 4 en 5** speculeren wij dat de osteoblast-gemedieerde osteoclastogenese, geïnduceerd door serum van RA patiënten, gedeeltelijk veroorzaakt kan worden door CXCL8 en CCL20 in actief-RA serum. Dit maakt dat deze chemokines interessante “targets” zijn voor toekomstig onderzoek naar individuele behandelingsopties voor patiënten met RA die niet goed reageren op de bestaande behandeling.

Osteocyten zijn zeer mechanogevoelig: na mechanische stimulatie veranderen ze de productie van een reeks signaalmoleculen die de werving, differentiatie en activiteit van osteoblasten en osteoclasten moduleren. Daarom spelen de door de osteocyten geproduceerde cytokines en signaalmoleculen een cruciale rol in de botadaptatie. In **hoofdstuk 6** onderzochten we of actief RA-serum de intrinsieke capaciteit van osteocyten om mechanische stimuli waar te nemen kan beïnvloeden. Ook werd onderzocht of mechanische belasting van osteocyten het effect van actief RA-serum op de osteocyt-osteoclast communicatie kan beïnvloeden. We hebben gebruik gemaakt van primaire humane botcellen, die een mengsel zijn van osteoblasten en osteocyt-achtige cellen. Omdat osteocyten meer mechanogevoelig zijn dan osteoblasten hebben we de cellen als “osteocyten” bestempeld in **hoofdstuk 6**. We vonden dat actief RA-serum geen invloed had op

de intrinsieke capaciteit van osteocyten om mechanische stimuli waar te nemen. We konden aantonen dat actief RA-serum de genexpressie ratio van RANKL/OPG in osteocyten verhoogde, evenals de osteocyt-gemedieerde osteoclastogenese, en dat mechanische belasting het omgekeerde effect had. Deze bevindingen suggereren dat lichaamsbeweging, zoals zeker mogelijk is bij RA patiënten zonder pijnklachten, of andere vormen van belasting van bot, zoals vibratie platvormen, een therapeutische werking kunnen hebben ter preventie van osteoporose in RA of andere inflammatoire ziektes.

Activatie van de kanonieke Wnt “signaling” exclusief in osteocyten induceert bot anabolisme en prikkelt Notch “signaling”. Osteocyten hebben een functie in de fosfaat homeostase. Hiertoe produceren osteocyten een reeks aan cytokines en signaalmoleculen. In voorgaande hoofdstukken werd gebruik gemaakt van een mengsel van osteoblast-achtige en osteocyt-achtige cellen voor de experimenten, terwijl we het liefst een zo puur mogelijke kweek van humane osteocyten zouden gebruiken, bij voorkeur in hun eigen omgeving. Helaas is er een gebrek aan goede humane osteocyt-celmodellen om hun functie *in vitro* te bestuderen. In **hoofdstuk 7** hebben we humane bot chips gekweekt met daarin osteocyten ingebed in hun natieve matrix. We hebben het effect onderzocht van exogene recombinante cytokines, chemokines, en actief RA-serum op osteocyt “signaling”. We vonden dat exogene recombinante inflammatoire cytokines de genexpressie verhoogde van de cytokines IL-1β, IL-6, IL-8, TNFα, FGF23 (betrokken bij de fosfaathomeostase), en de Wnt antagonist SOST, terwijl de chemokine CCL20 de genexpressie van TNFα, IL-6, and IL-8 verhoogde in osteocyten. Evenzo verhoogde serum van actieve RA patiënten de genexpressie van IL-1β, TNFα, FGF23, SOST, en de Wnt antagonist DKK1 in osteocyten. Deze resultaten suggereren dat osteocyten een sleutelrol kunnen spelen in de regulatie van de botmassa gedurende systemische ontsteking en daarom zouden ze een uitstekende “target” kunnen zijn voor de preventie van botverlies bij inflammatoire ziektes.

In dit proefschrift hebben we aangetoond dat verdund (1:10) actief RA-serum al van invloed was op de osteoblast functie, osteoblast-osteoclast communicatie, osteochondrogene differentiatie van voorlopercellen, osteocyt “signaling” en osteocyt-osteoclast communicatie, wat aangeeft dat de effecten *in vivo* alleen maar nog sterker kunnen zijn. Zulke effecten kunnen de bothomeostase verstoren, hetgeen systemisch botverlies en verhoging van fractuurrisico *in vivo* veroorzaakt. Door mechanische stimulatie van osteocyten was het mogelijk om de effecten van actief RA-serum-gemedieerde toegenomen osteocyt-osteoclast communicatie af te zwakken. Dit suggereert dat mechanische belasting van osteocyten een mogelijke optie is voor de behandeling van botverlies bij RA. De resultaten zoals beschreven in dit proefschrift dragen bij aan de start van een nieuwe onderzoeksrichting in de osteoimmunologie. Immers regelmatig wordt door

te veel patiënten in de kliniek ervaren dat bestaande medicatie niet helpt om de ontsteking af te zwakken en botverlies te voorkomen. Dit kan op zijn beurt weer leiden tot de ontwikkeling van nieuwe medicatie tegen nieuwe “targets” en interventies, zoals antistoffen tegen CXCL8 en CCL20, die effectiever kunnen zijn in het afzwakken van het ontstekingsgerelateerde botverlies als bestaande medicatie hierin niet slaagt. Zulke medicijnen kunnen behulpzaam zijn bij het ontwerpen van meer geïndividualiseerde behandelingen tegen algemeen botverlies bij systemische inflammatie.

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Dankwoord

Dhanyabad

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About The Author

About the author

Janak Lal Pathak was born on 21st February, 1982 in Jhapa, Nepal. In 2000 he received his high school diploma from the Institute of Science and Technology, Amrit Campus, Tribhuvan University, Kathmandu, Nepal. Afterwards, he studied Bachelor in Medical Laboratory Technology at Institute of Medicine, Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal. From 2006-2007 he worked as a Laboratory quality control officer at Siddhi Polyclinic Research Lab, Kathmandu, Nepal. He was awarded by China Scholarship Council (CSC) fellowship from the Chinese government to follow Chinese language course (2007-2008) and perform MSc degree in clinical medicine, laboratory sciences (2008-2011). In 2011 he received European Commission PhD fellowship through MOVE-AGE, an Erasmus Mundus Joint Doctorate programme to do Doctoral research at Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and VU University Amsterdam, MOVE Research Institute Amsterdam, Amsterdam, The Netherlands, and Skeletal Biology and Engineering Research Center, KU Leuven, Leuven, Belgium. His PhD research resulted in this thesis entitled: “The Etiology of Generalized Osteoporosis in Rheumatoid Arthritis”. He is currently working on bone biology and osteoimmunology that can lead to the understanding of the mechanism of generalized bone loss in systemic inflammatory diseases. He is married to Lijuan Mao.

Publications

Papers

- **Pathak JL**, Bravenboer N, Verschueren P, Lems WF, Luyten FP, Klein-Nulend J, Bakker AD: Inflammatory factors in the circulation of patients with active rheumatoid arthritis stimulate osteoclastogenesis via endogenous cytokine production by osteoblasts. Osteoporos Int 2014;25:2453-2463.
- **Pathak JL**, Bravenboer N, Bakker AD, Verschueren P, Lems WF, Luyten FP, Klein-Nulend J: Mechanical loading reduces inflammation-induced human osteocyte-to-osteoclast signaling. Accepted for publication in Calcified Tissue International.
- **Pathak JL**, Bakker AD, Verschueren P, Lems WF,Luyten FP, Klein-Nulend J, Bravenboer N: CXCL8 and CCL20 enhance osteoclastogenesis via modulation of cytokine production by human primary osteoblasts (submitted).
- **Pathak JL**, Bravenboer N, Bakker AD, Verschueren P, Klein-Nulend J, Luyten FP: Serum of patients with active rheumatoid arthritis inhibits differentiation of osteochondrogenic precursor cells (submitted).
- **Pathak JL**, Bravenboer N, Bakker AD, Verschueren P, Lems WF, Luyten FP, Klein-Nulend J: Inflammatory cytokines affect the production of osteocyte-related signaling molecules by human bone cells cultured in their native matrix (in preparation).

Book chapter

- **Pathak JL**, Jansen IDC, Bravenboer N, Klein-Nulend J, Bakker AD: Biomechanics of bone cells. Book chapter in Experimental Research Methods in Orthopedics and Trauma, (Thieme; editors: R. Helmer, H. Simpson, P. Augat), chapter38, p.p. 323-331, 2015.

Awards and grants

- ECTS & IBMS New Investigator Award 2015, at the 4th Joint Meeting of European Calcified Tissue (ECTS) and International Bone and Mineral Society (IBMS), Rotterdam, The Netherlands, 25-28 April, 2015.
- Dutch Society for Calcium and Bone travel Grant 2015 to attend the 4th Joint Meeting of European Calcified Tissue and International Bone and Mineral Society, Rotterdam, The Netherlands, 25-28 April, 2015.
- MOVE Award 2015 for the best publication entitled “Inflammatory factors in the circulation of patients with active rheumatoid arthritis stimulate osteoclastogenesis via endogenous cytokine production by osteoblasts” at the 6th Annual MOVE Research Meeting 2015, Amsterdam, The Netherlands, February 4, 2015.
- Charles D. Tenney International Fellow to Participate School of Pharmaceutical Science and Technology (SPST’s) Symposium on Direction in Modern Pharmaceutical Science (SDMPS), Tianjin University, Tianjin, China, Dec 3-6, 2014.
- IBFF International Travel Award to attend 12th International Bone Fluid Flow (IBFF) workshop, Houston, Texas, USA, September 10-11, 2014.
- American Association for Clinical Chemistry (AACC) International Travel Grant to attend the AACC Annual Meeting in Atlanta, GA, USA, July 24-28, 2011.
- Gallwas 5 Year Membership Grant (2011-2015) for the American Association for Clinical Chemistry.
- Ditan International Travel Grant Award to attend the 4thDitan International Conference on Infectious Diseases, Beijing, China, July 15-18, 2010.

International meetings attended

2015	4th joint meeting of European Calcified Tissue Society and International Bone and Mineral Society, Rotterdam, The Netherlands (1 podium and 1 poster presentation)
2014	Annual Meeting of American society for Bone and Mineral Research, Houston, TX, US (2 posters presentation)
2014	12th International Bone Fluid Flow (IBFF) workshop, Houston, TX, US 2014 (podium presentation).
2014-2012	Move-Age Annual Conferences, Amsterdam, The Netherlands (podium presentation)
2011	Annual Meeting of American Association for Clinical Chemistry, Atlanta, GA, USA (poster presentation).
2010-2009	Ditan International Conferences on Infectious Diseases, Beijing, China (2009, 2010: poster presentation)
2005	Congress of Asia Pacific Association of Societies of Pathologists, Kathmandu, Nepal (podium presentation)

National meetings attended

2015-2013 MOVE Annual Meetings, Amsterdam (2013, 2014, 2015: poster presentation)

2014-2011 Annual Meetings of the Dutch Society for Calcium and Bone Metabolism (NVCB), Zeist (2012, 2013, 2014: podium presentation)

2013 7th VUmc Science Exchange Day, Amsterdam (poster presentation)

2013 Annual Meeting of the Netherlands Institute of Dental Sciences, Lunteren, (podium presentation).

Extracurricular Activities

- Organizer of Symposium "Bone cell communication and bone loss in rheumatoid arthritis" under auspices of the MOVE Research Institute Amsterdam, June 19, 2015, ACTA, Amsterdam, The Netherlands (co-organizers: N. Bravenboer, A.D. Bakker, J. Klein-Nulend).
- 24th Annual Meeting of the Dutch Society for Calcium and Bone Metabolism (NVCB), Zeist, The Netherlands, November 12-13, 2014. Oral Session Chair, Nov 12, 2014.
- Founder member of Manmohan Memorial Hospital (a community based hospital), Kathmandu, Nepal, 2007.
- Worked as an editor of Journal of Nepal Association for Medical Laboratory Sciences, Volume 7, Number 7; June 2005 and Volume 8, Number 8, January 2007.
- Founder member of Institute of Medical Education (an institute to train students who wants to pursue their future carrier in medical field), Kathmandu, Nepal, 2006.
- Worked as a joint secretary in fifth executive committee (2004) and Secretary in Sixth executive committee (2005) in Nepal Medical Laboratory Students' Society (NMLSS).
- Worked as a managing editor in scientific publication, SCI-MET (a yearly publication of Nepal Medical Laboratory Students' Society), Volume-6, Number-1, July 2005.

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